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1911-1960

H. D. Sisler

The death of Carroll Eastburn Cox on June 24, 1960, prematurely ended a distinguished career of service to plant pathology and to the science of botany in general.

Dr. Cox was born at Kennett Square, Penn., December 19, 1911. He received an A.B. degree from the University of Delaware in 1938, an M.S. degree from the Virginia Polytechnic Institute in 1940, and a Ph.D. degree from the University of Maryland in 1943. He was a member of the staff of the Department of Botany of the University of Maryland from 1943 until his death. He was appointed Professor of Plant Pathology in 1951.

Possessed of a fine personality and a broad background in plant pathology and botany, he was highly regarded as a teacher by his students and professional colleagues. Over the years he cheerfully suffered many inconveniences in devotion to research and teaching. Even in his final illness, during which time he retained his usual optimism, he assisted one of his graduate students in preparing his Ph.D. thesis and left his sick bed to attend the final oral examination.

On the campus he was noted for his work in faculty affairs. As chairman of the committee appointed by the president of the University in 1956 to draft a plan of faculty organization, he had major responsibility in developing the plan of organization presently in effect at the University of Maryland. Shortly before his death, he was cited by the faculty for his role in the plan of organization which has been highly successful during the past four years.

Dr. Cox was noted for his research on the mechanism of action of fungicides. His recent contributions on this subject appear in the Golden Jubilee Volume on Problems and Progress in Plant Pathology, and in the recent three-volume series on phytopathology published by the Academic Press, Inc.

He served as councilor and as editor for the American Phytopathological Society and as president of the Potomac Division of the Society. During the past year, he was the Society's representative to the American

Association for the Advancement of Science. He also served as president of the Botanical Society of Washington and as president of the University of Maryland chapter of Sigma Xi.

He was a member of The American Phytopathological Society, the Botanical Society of America, the American Association for the Advancement of Science, the Washington Academy of Sciences, Sigma Xi, and Phi Kappa Phi.

Dr. Cox is survived by his widow, Elizabeth, and his son, Julian, who continue to reside in College Park, Md.

PECTINOLYTIC AND CELLULOLYTIC ENZYME PRODUCTION BY PYTHIUM APHANIDERMATUM

N. N. Winstead and C. L. McCombs

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SUMMARY

Pythium aphanidermatum produced both pectinolytic and cellulolytic enzymes *in vitro* and *in vivo*. A depolymerase or endopolygalacturonase present in culture filtrates degraded sodium polypectate into smaller fragments but did not reduce the viscosity of citrus pectin solutions. No pectin methylesterase (PME) was produced by the organism in culture. Filtrates from diseased cucumber fruits contained PME and reduced the viscosity of both pectin and sodium polypectate solutions but did not hydrolyze these substrates to galacturonic acid. Extracts of healthy cucumbers contained PME, but no degradation of the 2 pectin substrates was observed. A partially heat-stable cellulolytic enzyme (Cx type) that reduced the viscosity of carboxymethylcellulose (CMC) but did not hydrolyze CMC to cellobiose was present in culture filtrates and in plant extracts of inoculated cucumbers. Cellobiose was degraded to glucose. The enzymes produced in culture did not macerate sweet potato tissues at pH 5.5 or pH 7.0. The softening of cucumber fruits affected with the cottony leak disease appears to be correlated with the activities of the specific pectinolytic and cellulolytic enzymes produced.

Introduction.—*Pythium aphanidermatum* (Edson) Fitz. incites a cottony-leak disease of cucumber, *Cucumis sativus* L., in North Carolina. The fungus is frequent in the fields but is of most importance as a transit disease (12). Economic losses in shipments are frequent when ambient daytime temperatures are 90-100°F (20). The symptoms of the cottony-leak disease in cucumbers are suggestive of active pectinolytic and/or cellulolytic enzymes.

The production of pectinolytic and cellulolytic enzymes by organisms and the role of such enzymes in pathogenesis and tissue disintegration have been the subject of many investigations. Husain and Kelman (18) reviewed much of this work and discussed the classification of pectinolytic and cellulolytic enzymes.

Although no reports were found concerning pectinolytic enzyme production by *P. aphanidermatum*, such production by *P. debaryanum* Hesse has been investigated extensively (1, 7, 8, 14, 16, 21). In this case no pectin methylesterase (PME) was detected but a pectin depolymerase (DP) was present. DP degrades pectin to large fragments without the production of mono-, di-, or tri-galacturonic acid (17, 27).

Except for that of *P. irregulare* Buisman (11), cellulase production *in vitro* by *Pythium* spp. has not been reported.

Softening of cucumbers in commercial brining vats has also been associated with pectinolytic enzymes (4, 10). This softening was recently attributed to the production of pectinolytic and possibly cellulolytic enzymes by fungi associated with flowers, ovaries, and fruit of pickling cucumbers (2, 3, 13, 23). Other studies have shown that pectinesterase is a naturally occurring enzyme in cucumber fruits (5). Etchells and coworkers (13, 23) have listed the fungi detected on cucumber fruit and flowers that produced pectinolytic and cellulolytic enzymes. Many of these organisms are pathogenic to cucumber; however, *Pythium aphanidermatum* was not included in these studies.

The role of pectinolytic and cellulolytic enzymes in tissue disintegration and in softening of cucumber salt stock has been demonstrated, but few investigations have been reported on the production of these enzymes by *Pythium* spp. Since the rapid softening and rotting of cucumbers infected with *P. aphanidermatum* suggests that such enzymes might be important in pathogenesis, the production of polygalacturonase (PG), PME, cellulase (Cx), and B-glucosidase by *P. aphanidermatum* were studied and are reported herein.

Materials and methods.—Used in all studies was a pathogenic isolate of *P. aphanidermatum* cultured from a diseased cucumber. Used in certain tests were 3 additional isolates (originally isolated from chrysanthemum, *Chrysanthemum morifolium* Ram.), obtained from F. A. Haasis of this station. Stock cultures were maintained in test tubes on oatmeal agar or potato-dextrose agar (PDA), and mycelium was transferred to the culture media.

The basal culture media for enzyme assays contained 3 g KH_2PO_4, 2 g K_2HPO_4, 0.5 g $MgSO_4$, 2 g asparagine, and water to make 1 L of media. Glucose, 5 or 12 g of carboxymethycellulose-C (70; Premium medium viscosity, Hercules Powder Co.), citrus pectin (Exchange, Lemon Products Company), sodium polypectate (California Fruit Growers Exchange), macerated filter paper, or cellobiose were used as carbohydrate sources. Media were adjusted to pH 6.0 with either NaOH or HCl and sterilized by autoclaving. Since cellobiose was hydrolyzed by autoclaving, it was sterilized by filtration (ultrafine fritted-glass filter). After growth for 3, 7, and 14 days in standing cultures, filtrates were assayed for activity of various enzymes. Controls were aliquots of media and culture filtrates boiled for 10 to 30 minutes.

Plant tissue samples for *in vivo* determination were obtained from inoculated slices of No. 2 or 3 cucumber fruit. Cucumbers were surface-sterilized with a 10% sodium hypochlorite solution, peeled, and sliced under aseptic conditions, placed in Petri plates, and inoculated with mycelium. Two to 4 days after inoculation, the cucumber slices were macerated, the homogenate was centrifuged, and the supernatant was

used for enzyme activity measurements. Controls were preparations from uninoculated tissue slices as well as boiled samples from inoculated tissue slices.

Enzyme assays were made as follows:

PME activity was determined by the bromthymol-blue indicator method described by Smith (24). A positive test for activity is indicated by a change in color of the solution from blue to yellow.

PG was assayed by measuring the loss in viscosity of 1.2% sodium polypectate or citrus pectin substrate in size 300 Fenske-Ostwald viscometers at 30±0.1°C (6). Activity was determined at pH 3.5, 5.5, and 7.0.

Cellulase Cx was determined by the loss in viscosity of a 1.2% solution of CMC at pH 5.5 at 30±0.1°C (6).

Filtrates were also assayed for cellulolytic enzyme activity (S factor) by the alkali cotton fiber-swelling test described by Marsh et al (19).

Chemical analysis of culture filtrates, substrates, combinations of filtrates and substrates, and extracts from healthy or infected cucumber slices were made by assaying various substrates after incubation with culture filtrates for reducing sugars by the dinitrosalicylic acid (DNSA) test described by Sumner and Somers (25).

Chromatographic analyses for hydrolytic products were made with 1-dimensional descending chromatograms. The sample was streaked on Whatman No.1 paper strips and irrigated for 16 hours with equal volumes of butanol-water (1250:84) and propionic acid-water (587:747) mixed just before use. The carbohydrates were detected by dipping the strips into solutions of anisidine-phosphate (22) or 2-aminobiphenyl (15). Known standards were cochromatogramed. Most tests were repeated at least 4 times.

Results.—Growth of all isolates in culture was always similar on a specific carbohydrate source. Each isolate made rapid growth on culture media containing either glucose or cellobiose; however, growth was slight on sodium polypectate, pectin, filter paper, and CMC and did not differ from that obtained on the basal medium, which contained asparagine but no carbohydrate. Growth on cucumber slices was very rapid, and all slices were completely rotted within 4 days.

Pectin methylesterase.—Culture filtrates were always negative for PME activity. Carbohydrate sources used in these tests were pectin, sodium polypectate, glucose, cellobiose, CMC, and the basal medium. Since growth on media containing pectin was poor, the fungus was grown in media containing both glucose and pectin. Growth was comparable to that observed on media containing glucose; however, no PME was detected after 7 or 14 days' growth. The supernatants from both inoculated and uninoculated cucumber slices exhibit PME activity. Test solutions became bright yellow within 3 hours. No quantitative difference in PME activity was apparent. No PME activity was detected in samples from heated extracts from inoculated and uninoculated cucumber tissues.

Table 1.—The % loss in viscosity of various substrates after 3 hours of incubation with filtrates from 14-day-old cultures of *Pythium aphanidermatum* on the basal medium plus sodium polypectate or glucose.

Substrate	pH	% loss in viscosity and culture carbon source[a]	
		Sodium pectate	Glucose
Sodium pectate	5.5	11	64
	7.0	9	71
Pectin	5.5	2	10
	7.0	1	7
Carboxymethylcellulose	5.5	26	61

[a] Values represent increase of viscosity loss over that of the boiled check.

Table 2.—The % loss in viscosity of various substrates after 3 hours incubation with extracts from cucumber slices uninoculated and inoculated.

Substrate	pH	% loss in viscosity and days after inoculation[a]		
		Uninoculated	Inoculated	
			2 days	4 days
Sodium pectate	5.5	0	16	69
	7.0	6	37	77
Pectin	5.5	4	20	57
	7.0	4	16	58
Carboxymethylcellulose	5.5	15	78	84

[a] Values represent increase of viscosity loss over that of the boiled checks.

Results were identical with each of the 5 isolates used.

Polygalacturonase.—PG activity varied with the medium and also with the substrate used in the viscosity determinations. Filtrates of cultures grown on pectin, sodium polypectate, filter paper, CMC, or on the basal medium exhibited slight PG activity when incubated on pectin or sodium polypectate. The reduction in the viscosity of sodium polypectate was always greater than that of pectin at both pH 5.5 and 7.0. PG activity of filtrates from cultures containing each of the media mentioned above were essentially of the same magnitude, and no marked differences were observed among filtrates from cultures grown for 3, 7, or 14 days.

Filtrates from cultures grown on media containing glucose as a carbon source markedly reduced the viscosity of sodium polypectate, but had only slight activity on the pectin substrates (Table 1). Filtrates from 3-day-old cultures reduced the viscosity of pectate at about the same rate as that recorded in Table 1 for the NaP media. Activity of filtrates from 7-day-old cultures was about ⅔ that of the 14-day-old cultures. Viscosity assays for PG activity with extracts from inoculated cucumbers differed from those of culture filtrates in that the viscosity of pectin substrates was reduced almost as much as the viscosity of the sodium polypectate substrates. The highest levels of PG enzyme activity from diseased cucumbers were detected 4 days after inoculation. The data in

Table 2 are for 1 of the 4 tests, since the results were essentially similar.

None of the culture filtrates or extracts from inoculated cucumbers reduced the viscosity of pectin or sodium polypectate at pH 3.5. PG activity of glucose culture filtrates and of inoculated cucumber supernatants was slightly more rapid at pH 7.9 than at pH 5.5.

Cellulase (Cx).—For any specific culture filtrate, reduction in the viscosity of CMC was usually greater than the reduction of the sodium pectate substrate. Cx activity of culture filtrates from 14-day-old cultures was much greater than that of 3- and 7-day-old cultures. Losses in CMC substrate viscosity were greatest when filtrates were prepared from cultures grown on glucose media. Activities of filtrates from media containing pectin, CMC, filter paper or the basal medium were essentially identical to those recorded for sodium pectate (Table 1). Extracts from cucumbers either 2 or 4 days after inoculation were more active than culture filtrates. Uninoculated cucumber slices were held 4 days before assay. In all cases extracts from such slices showed a slight reduction (5-20%) in the viscosity of CMC. Check samples were boiled for only 10 minutes and usually reduced the viscosity of CMC substrates from ⅓ to ½ as much as did unheated samples in preliminary tests. Hence, subsequent check samples were boiled vigorously for 30 minutes.

Cellulase (*S factor*).—All of culture filtrates were negative for the S factor.

Other isolates.—Assays were also made to determine PME, PG, and Cx activity by the chrysanthemum isolates of *P. aphanidermatum*. Cultures were grown on each of the carbon sources previously mentioned and on inoculated cucumber slices. PG activity was measured only as the loss of viscosity at pH 5.5. Results with these isolates were essentially similar to those previously recorded for the cucumber isolate.

Analysis of hydrolytic products.—Chromatograms were developed from most samples that showed even slight losses in viscosity. Heated samples of the culture filtrate and uninoculated media were run as checks. When sodium polypectate substrate was incubated with sodium polypectate culture filtrates, a trace of galacturonic acid was observed only 4 times; however, this trace was observed in 3 instances in the heated check and only once in a parallel unheated sample. No galacturonic acid was observed when filtrates were incubated with pectin substrate. Two distinct unidentified spots were present on chromatograms of sodium polypectate substrate incubated with filtrates from inoculated cucumbers. Both had low Rf values, and neither was galacturonic acid. No galacturonic acid was detected even after 24 hours of incubation. When CMC substrate was incubated with culture filtrates, no cellobiose was observed except when cellobiose was used as a carbon source in the original culture medium. Dextrose and fructose were observed on the chromatograms when either dextrose, cellobiose, or cucumber extracts (inoculated or uninoculated) were present in the samples. Filtrates from cultures grown on media containing cellobiose (sterilized by filtration) were used to determine whether cellobiose was converted to glucose. Growth on cellobiose media was comparable to that on dextrose medium. When filtrates of cultures grown on cellobiose medium were chromatographed, large quantities of glucose were detected. No traces of cellobiose were observed with 20 µl and only a trace of cellobiose was observed when 50 µl were spotted. Uninoculated samples of cellobiose media contained cellobiose but no glucose, indicating that most of the cellobiose in the uninoculated media had been converted to glucose.

Tissue maceration.—Samples of culture filtrates were taken from cultures grown on media containing glucose as a carbon source and incubated for 48 hours under toluol on sweet-potato disks 1 cm in diameter and about 2 mm thick. Disks placed in water, culture filtrate, or heated culture filtrate at both pH 5.5 and 7.0 appeared identical macroscopically. No maceration of any disks was evident.

Free-hand sections of cucumber tissues were examined microscopically at various stages of disease development. No evidences of maceration or cell separation were observed even in well-rotted tissues showing advanced symptoms of wet rot.

Discussion.—Growth with each of the carbon sources except glucose and cellobiose was limited and did not differ significantly from that on the basal media containing asparagine but no carbohydrate. This suggests that most of the growth on these media was associated with the utilization of asparagine, which was common to all media.

The PG of *P. aphanidermatum* apparently does not differ markedly from that of many other disease-inciting organisms (18). The PG or DP enzyme of *P. aphanidermatum* reduced the viscosity of sodium polypectate solution, but not that of pectin solutions unless an external source of PME was added. This PG differed from that reported for *P. debaryanum*, which rapidly reduced the viscosity of pectin solutions but not that of sodium polypectate solutions (1, 16, 17, 27). According to the classification of Demain and Phaff (9), the enzyme produced by *P. aphanidermatum* would be classified as endopolygalacturonase whereas the enzyme produced by *P. debaryanum* would be classified as endopolymethylgalacturonase. The pectic enzyme systems of these 2 *Pythium* spp. also have certain similarities. The PG of neither species hydrolyzed pectic substances to galacturonic acid, and neither species produced PME.

The presence of PME in cucumber fruits readily explains the loss in viscosity of pectin substrates incubated with filtrates from diseased cucumbers. This work verifies a previous report by Bell et al (5) that PME is produced in healthy cucumber fruit.

Unlike *P. irregulare* (11), *P. aphanidermatum* did not appear to utilize cellulosic materials as a carbon source, though *P. aphanidermatum* did hydrolyze

cellobiose to glucose. The cellulosic enzyme system produced by *P. aphanidermatum* is apparently of the Cx type but did not convert CMC to cellobiose. The hydrolytic products of CMC were not identified with the techniques used. The partial stability of this Cx enzyme at high temperatures is apparently unusual. Strider (26) reported that a commercial cellulase (enzyme 19, mix 12, Rohm & Haas) and the Cx enzyme produced by a *Penicillium* sp. were also partially heat-stable.

The production of endopolygalacturonase and Cx by *P. aphanidermatum* does not appear to be adaptive, since each was produced in media containing glucose. The enzymes produced *in vivo* and *in vitro* appeared essentially identical.

Symptoms of the cottony leak disease of cucumber appeared to be correlated with the activities of the pectinolytic and cellulolytic enzymes produced. Tissues became soft, yet little tissue disintegration and maceration were observed. Similarly the endopolygalacturonase and Cx enzymes produced by *P. aphanidermatum* reduced the viscosity of their respective substrates, yet they did not hydrolyze them to galacturonic acid and cellobiose. Organisms known to produce exopolygalacturonase or exopolymethylgalacturonase usually cause extensive tissue disintegration, reduce the viscosity of pectinolytic substrates, and hydrolyze these substrates to galacturonic acid (18). Hence, the lack of cell separation and tissue disintegration appears associated with the specific pectinolytic and cellulolytic enzymes produced by *P. aphanidermatum*.—Departments of Plant Pathology and Horticulture, North Carolina State College, Raleigh.

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A RAPID GAS CHROMATOGRAPHIC METHOD FOR DETERMINING FUMARIC ACID IN FUNGUS CULTURES AND DISEASED PLANT TISSUES

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SUMMARY

Excellent separation of the diethyl esters of fumaric, succinic, malonic, maleic, and malic acids were obtained on butanediol-succinate polyester or diethyl glycol succinate gas chromatographic columns. A quantitative procedure based on the gas chromatographic method was developed and used in the analysis of fumaric acid in cultures of *Rhizopus* spp. grown on an almond hull medium, in hulls rotted by *Rhizopus* on orchard trees, and in healthy and diseased leaves of almond affected by *Rhizopus*.

Fumaric acid was recently discovered to be a toxin responsible for twig dieback in the hull rot disease of almond as caused by *Rhizopus* spp. (3). During that study, a rapid and quantitative gas chromatographic method was developed for determining phytotoxic amounts of naturally occurring fumaric acid in diseased tissues.

Available methods involving enzymes (5), paper and column chromatography (1, 2), and permanganate titration (6) lacked the ease and applicability of the gas chromatographic method.

The present research was conducted to ascertain both the accuracy of the gas chromatographic method in determining diethyl fumarate and its applicability to analyses of fumaric acid in cultures of pathogenic species of *Rhizopus* and in diseased and healthy tissues of almond.

Materials and methods.—*Preparation of cultures.*—Single-spore isolates of *Rhizopus stolonifer, R. circinans, and R. arrhizus,* all pathogenic on almond fruits, were grown on potato-dextrose agar for 2 weeks. Sporangiospores from each culture were suspended in distilled water and adjusted to the same spore density with a B & L Spectronic 20 colorimeter. One-ml amounts of the spore suspensions were used to seed experimental flasks containing a water extract of almond hulls (1 kg of fresh hulls of the almond variety Ne Plus Ultra was blended for 2 minutes in 3 liters of distilled water). The suspension was heated 10 minutes at 80°C, and filtered with Whatman No. 1 paper. The filtrates (50 and 25 ml) were pipetted into 250-ml Erlenmeyer flasks and autoclaved 20 minutes at 121°C. Final pH was 4.8. The experimental cultures in the seeded medium were grown on a reciprocating shaker at 25–27°C. There were 2 similar experiments: 3 species of *Rhizopus* were grown on 50 ml of medium, harvested at 5-, 10-, and 15-day intervals, and filtered with Whatman No. 1 paper; and *R. circinans* was grown on 25 ml of medium and harvested at intervals of 24, 48, 72, 96, and 120 hours. One-ml aliquots of the culture filtrates were then analyzed for fumaric acid.

Preparation of plant extracts.—Fumaric acid was extracted from diseased and healthy leaves and hulls of almond by blending the tissues for 2 minutes in 70% ethanol. About 1 g (dry wt) of tissue was extracted with 10 ml cold ethanol, then extracted twice again (1 g tissue/5 ml ethanol). In earlier experiments, the combined extract was purified on an Amberlite IRA-400 (Rohm & Haas Co.) resin column according to the method of Bryant and Overell (1). Later, however, it was found that the ethanol extracts could be analyzed without the resin treatment.

Preparation of esters.—One-ml aliquots of culture filtrate or ethanol extract of plant tissue were air-dried in a 15-mm × 15-cm Pyrex test tube and dissolved in 5 ml of 4% v/v H_2SO_4–absolute-ethanol solution. The tube was capped with a glass marble and refluxed for 1 hour at 90°C. The mixture was then concentrated under an air stream or by heat to 2 ml and diluted with 4 ml of distilled water. One-half to 1 ml of *n*-heptane was then mixed thoroughly with the reaction mixture to extract the diethyl esters. During extraction, a black precipitate frequently formed at the interface of the heptane and water layers, making it difficult to obtain clear samples of the heptane solution. The heptane layer was easily clarified, however, by transferring the entire mixture to a stoppered 6 × 100-mm tube and centrifuging it for 15 minutes at 3000 G.

Per cent recovery of known fumaric acid from biological extracts was determined by adding 1.25 mg of fumaric acid/ml of extract before esterification.

Sensitivity of the method was determined by diluting aliquots of diethyl fumarate (Distillation Products Corp.) with *n*-heptane.

Gas-liquid chromatography.—After preparation of the esters, 10–60-μl aliquots of the heptane solution were analyzed with an Aerograph Model 100-C gas chromatograph. Either butanediol-succinate polyester (Craig polyester) or diethyl glycol succinate (DEGS) was used on crushed brick in 1/4-in. columns 5–7.5 feet long under the following conditions: Oven temperature, 150°C; helium flow rate, 150 ml/min; filament current, 250 ma; and recorder sensitivity, 1 mv. Both columns gave excellent resolution of mixtures of diethyl fumarate, diethyl maleate, diethyl succinate, diethyl malate, and diethyl malonate; however, the Craig polyester column was used mainly for the analyses reported in this paper. The retention times of the diethyl esters of various organic acids (Distillation Products Corp.) are given in Table 1. These values are ratios of the time required for the various esters to pass through the column to the time required for diethyl malonate (which was added to

Table 1.—Separation of esters of organic acids by gas-liquid chromatography.[a]

Ester	Retention time	
	Craig polyester	DEGS
Diethyl malonate	1.00	1.00
Diethyl fumarate	1.48	1.30
Diethyl succinate	1.53	1.45
Diethyl maleate	2.06	2.04
Diethyl malate		6.81

[a] Aerograph Model A-100-C; oven temp. 150°C; helium flow rate, 150 ml per minute; filament current, 250 ma.

the unknown samples) to pass through the column. Time retention values were used in tentative identifications of unknown compounds. Diethyl fumarate was positively identified by comparing the infrared absorption spectrum of the unknown substance (collected from the gas chromatography column in CS_2) with that of the known ester using a Perkin-Elmer 221 Spectrophotometer.

Quantitative analyses of fumaric acid in the biological samples were made by comparing the peak height registered on the recorder by diethyl fumarate with a standard curve obtained with known amounts of this ester (Fig. 1). Standards were run with each series of analyses of unknown samples. Peak height rather than peak area was used for quantitative determinations since plots of peak area were not linear; the relationship was linear for height values for concentrations of 10–210 μg of diethyl fumarate at 1 mv recorder sensitivity. The linear relationship was extended to 450 μg by attenuating the recorder sensitivity.

Results and discussion.—*Accuracy of the method.*—The efficiency of esterification was about 78% based on comparisons of peak heights of known concentrations of diethyl fumarate with those of the ester produced from known concentrations of fumaric acid.

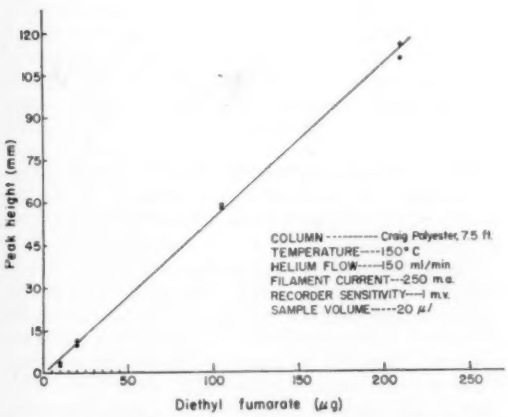

Fig. 1.—Standard curve showing a linear relationship between different concentrations of diethyl fumarate.

However, when known amounts of fumaric acid were added to biological extracts and then esterified, recovery was 102-104% (Table 2), based on the peak height of a known amount of fumaric acid esterified and analyzed in the same manner as the test solution. Quantitative recovery was obtained when as little as 10.5 μg of diethyl fumarate (7.1 μg fumaric acid) in the test sample was injected into the gas chromatograph.

Table 2.—Recovery of fumaric acid when added to a known aliquot of culture filtrate of *Rhizopus arrhizus*.[a]

1 ml culture filtrate	1 ml culture filtrate + 1.250 mg fumaric acid	Theoretical recovery	Per cent recovery
0.865 mg	2.177 mg	2.115 mg	102
0.909 mg	2.259 mg	2.159 mg	104

[a] Figures represent the amount of fumaric acid determined as the diethyl ester using gas-liquid chromatography. Each value is an average of 2 analyses.

The concentration of fumaric acid in biological extracts was determined by using a standard curve of peak heights whose relationship was linear between 10 and 210 μg of diethyl fumarate (Fig. 1). Fumaric acid and its cis-isomer maleic acid, were easily separated by this method. Excellent separations also occurred for esters of malic acid, fumaric acid, succinic acid, and malonic acid in mixtures; however, the solubility of diethylmalate in water caused a poor recovery of this ester from the reaction mixture with *n*-heptane. Similar methods for quantitative determinations of succinic and malic acids in biological extracts will be reported elsewhere.

Production of fumaric acid by Rhizopus.—Fumaric acid was produced in various amounts by the 3 pathogenic species of *Rhizopus* obtained from almond hulls (Fig. 2). The amounts are given in μg per ml of culture filtrate per mg dry weight of fungus mycelium (Fig. 2) to correct for differences in growth rates between the isolates. Total fumaric acid production by these isolates is more apparent, however, when it is expressed as mg per ml of culture filtrate. Thus, respective accumulations from *R. circinans*, *R. stolonifer*, and *R. arrhizus* were 1.3, 1.0, and 0.6 mg/ml at 5 days, 2.2, 1.1, and 0.7 mg at 10 days, and 2.0, 1.46, and 0.9 mg at 15 days.

Table 3.—Concentration of fumaric acid in leaves and fruits of almond.

Treatment	Fumaric acid (μg/g dry wt.)
Hulls rotted by *Rhizopus* spp. in orchard trees	225.8
Control	trace
Detached hulls rotted by *Rhizopus* spp. in laboratory	461.0
Control	trace
Blighted leaves on orchard trees	trace
Healthy leaves	trace

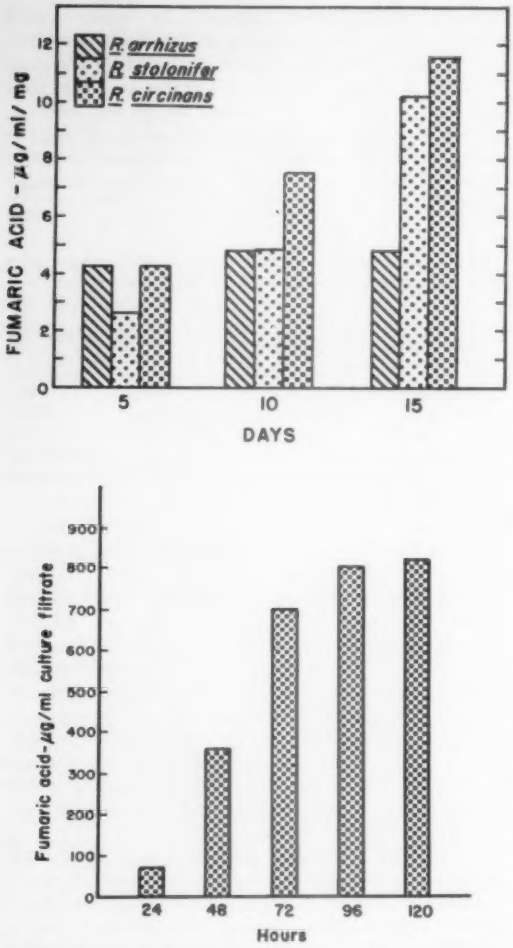

Fig. 2–3.—2) Production of fumaric acid in cultures of different age of 3 species of *Rhizopus*. Fumaric acid is expressed in µg per ml of culture filtrate per mg (dry wt) of mycelium. **3)** Production of fumaric acid by *Rhizopus circinans*.

Variability was apparent among the isolates in accumulation of fumaric acid in the culture medium, and further experiments indicated that fumaric acid production in cultures of *R. circinans* was almost linear for the first 72 hours, then gradually decreased (Fig. 3). Thus, it was clearly shown that almond hull medium supported excellent growth of *Rhizopus* and that large amounts of fumaric acid were produced within 72 hours.

Fumaric acid in diseased and healthy almond leaves and hulls.—Analyses of fumaric acid in almond tissues showed a high concentration of this acid in rotted hulls, in contrast to trace amounts in healthy hulls; both blighted and healthy leaves contained trace amounts of fumaric acid (Table 3). The significance of these results in relation to other analytical data as they relate to the physiological basis of almond hull rot will be more fully discussed in a later paper. Preliminary reports on these findings have been made (3, 4).—Department of Plant Pathology, University of California, Davis.

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BOOK LISTINGS

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This book describes the methods, techniques and principles of the Institute. This is done in 13 chapters: The Commonwealth Mycological Institute, by S. P. Wiltshire; The herbarium, by M. B. Ellis; How to describe a fungus, by F. C. Deighton; Laboratory methods used in the cultural collection, by H. A. Dade; The treatment of important pathogenic Phycomycetes, by Grace M. Waterhouse; Bacteria and the herbarium, by A. C. Hayward; Collecting fungi, by C. Booth; Collecting in the tropics, by F. C. Deighton; Collecting in the clinic, by G. C. Ainsworth; The "Index of Fungi," by F. C. Deighton; The "Bibliography of Systematic Mycology," by Grace M. Waterhouse: The Herbarium and The "Review of Applied Mycology," by E. B. Martyn; and The "Review of Medical and Veterinary Mycology" and The Herbarium, by G. C. Ainsworth.

Plant Pathology Laboratory Manual. *Fulton, J. P., D. A. Slack, N. D. Fulton, J. L. Dale, M. J. Goode, and G. E. Templeton.* 1960. 2nd ed. Burgess Publishing Co., Minneapolis, Minnesota. 95 p. $3.00.

This manual is divided into 10 parts. The first part contains directions for use of the microscope in preparing mounts for microscopic examination, and for recording experiments. Koch's postulates are explained in this part. Chapters 2–9 describe representative diseases caused by bacteria, the plasmodiophorales, phycomycetes, ascomycetes, fungi imperfecti, basidiomycetes, nematodes, and viruses. A glossary of terms is given in part 10.

PECTOLYTIC ENZYMES OF CERATOCYSTIS FIMBRIATA

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SUMMARY

Ceratocystis fimbriata was shown to produce two kinds of pectin depolymerase and a cellulase. Depolymerase A decomposed polypectate more rapidly than pectin and showed a pH optimum at 5.8. Depolymerase B decomposed pectin, but not polypectate, with maximum activity at pH 8.1. The cellulase decomposed carboxymethyl cellulose. Depolymerase A and cellulase were shown to be produced in the infected tissue of sweet potato. The pathogen produced neither pectin methyl esterase nor exo-pectinase in detectable amounts. An inhibitive factor of the depolymerases which seemed to be a protein, was found in sweet potato tissue. Some abnormal metabolites in the diseased sweet potato, such as chlorogenic acid, umbelliferon, and ipomeamarone, did not significantly inhibit the two depolymerases.

Introduction.—In the laboratories of Stahmann and Walker (9, 10, 14, 25) it has been observed that *Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyd. & Hans., the pathogen of tomato wilt, secretes an extracellular pectin depolymerase that causes pectin plugs to form in the vascular system and wilt symptoms to develop. When inoculated onto isolated tissue of tomato stem, the pathogen grew equally well on tissues from resistant and susceptible plants, but depolymerase activity was 3 times as great on susceptible tissue as on resistant (3). With the same method, it was found that the pectin methyl esterase activity of a related pathogen, *F. oxysporum* f. *pisi* (Lindford) Snyd. & Hans., Race 1, was greater on susceptible pea tissue than on resistant (27).

On the other hand, it is well known that symptoms of soft rot diseases are largely due to cell maceration by pectolytic enzymes produced by pathogens. Wood's group (8, 17, 28) has done research on the mechanism of soft rot caused by some pathogenic fungi and bacteria, and Walker's group (7, 19) clarified the relationship of pectic enzyme production to soft rot of stored vegetable organs induced by *Sclerotinia sclerotiorum* and *Rhizopus stolonifer* (Ehr. ex Fr.) Lind.

One approach to study of the mechanism of pathogenicity and resistance of the host is investigation of the relation between pathogenicity and such hydrolytic enzymes as depolymerase in a wide range of plant diseases. Therefore, cell-wall-decomposing enzymes produced by *Ceratocystis fimbriata*, Ellis and Halstad, the fungus causing black rot of sweet potato, were studied. This disease is a dry rot, quite different from wilt or soft rot.

Studies of the hydrolytic enzymes may be especially valuable in clarifying two problems:

The first is the exact role of the enzymes in the penetration of the pathogen into the host cells. From investigations of several cultural media on which *C. fimbriata* was grown, and anatomical observations of the diseased tissue of sweet potato (24), it was assumed that the pathogen did not produce toxic low-molecular compounds in the host cells, or if so, the compounds must be weak in toxic action or negligible in amount. On the other hand, it might produce hydrolytic enzymes to decompose the cell wall and facilitate penetration.

The second problem concerns participation of such enzymes in ipomeamarone formation. When roots are infected, ipomeamarone (a sesquiterpene) and similar metabolites are formed as a biochemical symptom (1, 15, 21, 22). In resistant varieties their formation is limited to infected tissue; but in susceptible varieties, it is also seen in tissue just adjacent to the infected area. There is a possibility that the formation is induced by disturbance of normal metabolism by some products of the pathogen, such as pectolytic enzymes, which have slow diffusion rates.

Materials and methods.—*Pectin methylesterase activity.*—Measurement of pectin methylesterase activity was in terms of the increase in acidity on hydrolysis of pectin, expressed as microequivalents (μeq.) of the released methoxy groups (27). Ten ml of 1.5% pectin solution (pH 5.5) were incubated with 2 ml of 0.5*M* acetate buffer (pH 5.5) and 3 ml of enzyme solution for 90 or 150 minutes at 30°C. As a control, the heated enzyme solution was used in place of the enzyme solution. After incubation, the mixture was titrated to pH 7.7 with 0.025*N* sodium hydroxide. Microequivalents of methoxy groups released were calculated from the difference between the titration volume of the enzyme mixture and that of the control.

Reduction in viscosity by pectolytic enzyme.—Reduction in viscosity was determined by measuring the % reduction in viscosity of polypectate solution (3). Three ml of 1% sodium polypectate (pH 4.5), and 3 ml of the enzyme solution were mixed at 30°C. Five ml of the mixture were put into an Ostwald viscosimeter, and viscosity was measured intermittently. Zero time for the reaction was taken as the time when the enzyme solution was added to the substrate. Flow at 0 time was obtained by extrapolation. Percentage

reduction in viscosity (A) was expressed by the following equation:

$$A = \frac{T_{cont.} - T}{T_{cont.} - T_{H_2O}} \times 100$$

in which $T_{cont.}$ = flow time in seconds at 0 time in the control reaction mixture with the heated enzyme; T = flow time of the reaction mixture at time T; and T_{H_2O} = flow time of the mixture in which water was substituted for the substrate.

Liberation of reducing groups by pectolytic enzyme.—The formation of reducing groups, determined by iodometric titration, is expressed as μeq of reducing groups liberated from polypectate (3). Fifteen ml of 1% polypectate (pH 4.5), 1 ml of 0.5*M* acetate buffer (pH 4.5), and 4 ml of the enzyme solution were mixed at 30°C. Immediately after, 5 ml of the mixture were added to 2 ml of 1*M* sodium carbonate for assaying reducing groups at 0 time. The remaining mixture was incubated 90 or 180 minutes at 30°C. Then 5 ml of the mixture were added to 2 ml of 1*M* sodium carbonate, mixed with 5 ml of 0.1*N* iodine solution, and after 30 minutes mixed with 4 ml of 2*M* sulfuric acid. The mixture was titrated by 0.05*N* sodium thiosulfate. The difference in titration volume between 0 time and the final stage was used for calculation of the activity.

Cellulase activity.—Cellulase activity was estimated by determining the % reduction in viscosity of a carboxymethyl cellulose solution (3). Three ml of 1.25% carboxymethyl cellulose (MC-70-Medium, D.S. 0.7) were mixed with 1 ml of 0.5*M* citrate buffer (pH 5.0) and 3 ml of the enzyme solution at 30°C. After vigorous shaking 5 ml were put into an Ostwald viscosimeter for viscosity measurements. An enzyme solution heated for 20 minutes in boiling water was used as a control to check nonenzymatic change in viscosity. Percentage reduction in viscosity (A) was calculated by the following equation:

$$A = \frac{T_{cont.} - T}{T_{cont.} - T_{H_2O}} \times 100$$

in which $T_{cont.}$ = flow time of the reaction mixture with the heated enzyme; T = flow time of the reaction mixture at time T; and T_{H_2O} = flow time of the mixture containing water in place of the substrate.

pH-activity curves.—For investigation of pH-activity curves of pectolytic enzymes, McIlvaine's buffer (0.25*M* citric acid and 0.5*M* disodium phosphate) and borax buffer (0.25*M* borax and 0.5*M* monopotassium phosphate) were used, in the respective pH ranges of 4.0–8.0 and 8.0–9.0. Reduction in viscosity was measured as above. For reducing-group determinations, the reactants were used at the same ratio as for viscosity measurement, i.e., 8 ml of 1% polypectate or pectin, 2.67 ml of the buffer, and 8 ml of the enzyme solution were mixed and incubated. That made possible a comparison of both activities.

Activity units of the enzymes.—*C. fimbriata* produced 2 kinds of pectolytic enzymes, depolymerase A (DP-A) and B (DP-B). The reaction mixture used for estimating the depolymerase activities was almost the same as for determining reduction in viscosity. For DP-A it was 3:1:3 (v/v) 1% sodium polypectate (pH 5.8), 0.5*M* acetate buffer (pH 5.8), and enzyme solution, and for DP-B it was 3:1:3 (v/v) 1% pectin (pH 8.1), McIlvaine buffer (pH 8.1), and enzyme solution plus distilled water. For both enzymes the concentration that showed 50% reduction in viscosity at 60 minutes was defined as one unit of activity. One unit of activity showed 34.3 and 66% of the reduction at 30 and 120 minutes, respectively. The activity of any enzyme sample was estimated by the following procedures: % reduction in viscosity at 30, 60, and 120 minutes was determined and converted to activity units by using curves showing the relation between activity units and % reduction in viscosity at 30, 60, and 120 minutes (Fig. 1). The mean of 3 values was selected. The percentage reduction in viscosity was proportional to the logarithm of activity (log E) within a certain range of the reduction. This log relationship is shown in Fig. 2. Saito (18) obtained similar results on enzymes from other sources.

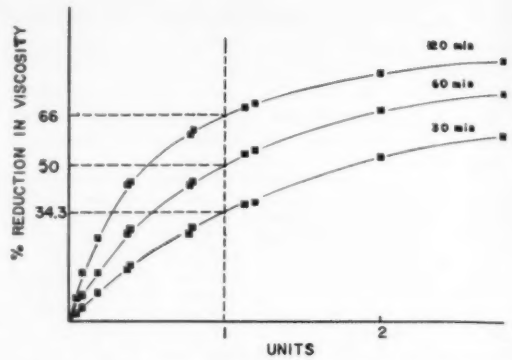

Fig. 1.—Relationship between reduction in viscosity and activity units of depolymerase A.

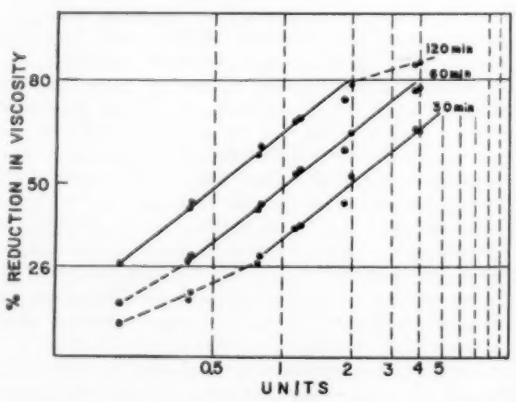

Fig. 2.—Relationship between reduction in viscosity and logarithm of activity units of depolymerase A.

Percentage reductions shown by DP-A and DP-B are proportional to log E in the respective ranges of 20–80% and 30–75%. Therefore, activity units were obtained from the figures in such ranges (Fig. 2). Fig. 1 and 2 show only the figures for DP-A since those for DP-B are almost the same.

Three enzyme extracts for assaying pectolytic activity of C. fimbriata.—Enzyme extract A.—Two hundred g of sliced potato tuber tissues were homogenized with 500 ml of distilled water in a Waring blender. The homogenate was filtered through cheesecloth, boiled gently, and filtered through filter paper. Ten g of pectin and 20 g of glucose were dissolved in warm water, and pH was adjusted to 5.8 with 3*N* NaOH. Both solutions were mixed, and the volume was adjusted to 1 L. Fifty ml of the solution were put into 500-ml Erlenmeyer flasks and sterilized in an autoclave. The medium was inoculated with spores of *C. fimbriata*, and the fungus was grown with shaking for 1 day at 25°C. and then under still culture for 4–9 days at 28°C. Mycelium covered the surface and formed perithecia within 4 days of still culture, and ascospores came out from perithecia between 5 and 9 days. After 4 or 9 days of still culture, the mycelium was taken out, and the medium centrifuged 10 minutes at 4000 rpm and filtered through filter paper. The filtrate was used as enzyme extract A. For the comparison, *F. oxysporum* f. *lycopersici* was grown under the same conditions as *C. fimbriata*, and the enzyme was extracted from the cultural medium in the same way.

Enzyme extract B.—Casein hydrolysate (0.2%) and thiamine (10μg%) were added to the potato extract, in addition to 2% glucose and 1% pectin. Final pH was adjusted to 6.0. The cultural medium was inoculated with spores of *C. fimbriata* or *F. oxysporum* produced in potato extract containing pectin by shaking for one day, and the fungi were grown 7 days under still culture at 28°C. Enzyme extracts were made with the same method as for extract A. *F. oxysporum* was also grown on the Czapek-Dox solution containing 2% glucose and 1% pectin.

Enzyme extract C.—Five g of wheat bran were mixed with 10 ml of distilled water in each 500-ml Erlenmeyer flask, sterilized, inoculated with spores of *C. fimbriata* or *F. oxysporum* (produced as shown for extract B), and left 5-8 days at 28°C. The growth of *C. fimbriata* was much inferior to that of *F. oxysporum* and wheatbran turned black-brown, perhaps from the action of laccase produced by the fungus. After the growth period, 50 ml of distilled water and 10 drops of toluene were added to each medium, and the mixture shaken gently overnight. The suspension was filtered through cheesecloth, and the filtrate was centrifuged. The supernatant was used as enzyme extract C.

Extraction of pectolytic enzyme produced by C. fimbriata on sweet potato tissue.—Usually, after infection of sweet potato tissue by *C. fimbriata*, secondary saprophytic microorganisms grow in the infected tissue. To test the activity of pectolytic enzyme produced by the pathogen in the host tissue, the tissue should be kept free of such secondary microorganisms. Two sterilizing methods were investigated. One was to expose sweet potato roots to propylene oxide vapor. This treatment damaged the tissue and caused bacteria to grow in the injured part. The other was to dip the roots into 10% Klenzade (0.64% sodium hypochlorite) for 30–60 minutes, wash them, and cut them into several slices. This treatment prevented contamination by bacteria without damaging tissue. The pathogen grew well on the surface and penetrated the tissue.

Enzyme Extract I.—Spores produced in shake cultures on pectin-containing medium were collected by centrifuge, washed with sterilized distilled water, recentrifuged, suspended in distilled water, and inoculated onto cut surfaces of roots of the susceptible sweet potato variety Porto Rico. Two days after the inoculation, the pathogen penetrated the tissue 1 mm deep, the mycelium covered all the surface, and the strong resistant action was not seen in the tissue. Fourteen g of the infected tissue (including some fungal tissue) as well as 14 g of adjacent healthy tissue were homogenized with 28 ml of 0.05*M* phosphate buffer (pH 6.7) in a mortar and pestle at 4°C. Each homogenate was filtered through cheesecloth and centrifuged 25 minutes at 12,000 rpm. Each supernatant was dialyzed against 0.02*M* phosphate buffer (pH 6.7). Three ml of each dialysate were used for measurement of reduction in viscosity, as enzyme extract I. The pH of the reaction mixture was 6.3.

Enzyme extract II.—The mycelium grown by still culture on pectin-containing medium was washed with sterilized distilled water and ground gently with water. The suspension was used as an inoculant. The methods for growing the pathogen and making the enzyme extracts were the same as for enzyme extract I.

Enzyme extracts III and IV.—The spore suspension for inoculation on the tissue was prepared as for enzyme extract I. Three days after the inoculation, 8 g each infected tissue and adjacent healthy tissue were homogenized 30 seconds with 100 ml of cold acetone in a Waring blender. The homogenates were filtered through filter paper, and the residues were washed with 100 ml of cold acetone and cold ether. The acetone powders were dried in a vacuum desiccator over phosphorus pentoxide, and homogenized with a mortar and pestle in 19 ml of 0.02*M* phosphate buffer (pH 6.6). The homogenates were filtered through cheesecloth and centrifuged 25 minutes at 12,000 rpm. The supernatants were dialyzed against the buffer before assay.

Enzyme extracts for pH-activity curves and other experiments.—The wheat bran extract was found to contain the highest activity of depolymerase, and to be most appropriate for further experiments. To remove any inhibitors or stimulators, the crude extract was saturated with ammonium sulfate, left in cold for several hours, and centrifuged 30 minutes at 4500 rpm. The precipitate was dissolved in a small volume

of distilled water, dialyzed against distilled water, and centrifuged 20 minutes at 9000 rpm (stock solution A). Sometimes the following procedure was used: To the supernatant was added dilute hydrochloric acid to pH 4.28, the solution was centrifuged 30 minutes at 10,000 rpm, and the supernatant was dialyzed against distilled water (stock solution B). The enzyme extracts thus obtained had 30 times as much activity as the original extract when measured by reduction in viscosity. Stock solutions A and B were kept frozen, and diluted to suitable concentration for assaying.

Results.—*Pectin methyl esterase activity.*—The 3 enzyme extracts (A, B, C) from cultural media in which *C. fimbriata* was grown were assayed for PME activity, but no activity was found. In contrast, activity was high for extracts from the same 3 cultural media in which *F. oxysporum* was grown (Table 1).

It was possible that pectin methyl esterase activity might not have been found if the optimum pH of the enzyme were in the alkaline range, though pectin methyl esterase having a pH optimum in the alkaline range has not yet been detected in such fungi (4, 6). Therefore, using preparations of the concentrated enzyme, assays were made at 2 pH values, 5.5 and 8.1; the amount of methoxy groups found was negligible in both cases, even though the extract was concentrated more than 5 times and the reaction was carried out for 90 minutes. We concluded that *C. fimbriata* does not produce detectable pectin methyl esterase.

Polypectate decomposition.—Both the liquid cultural media and the wheat bran extract of *C. fimbriata* showed high activity in reducing the viscosity of polypectate solution. The activity of the liquid cultural media was a little less and that of the wheat bran extract was much higher than with *F. oxysporum*. Activity measured by reducing-group production was lower than that by viscosity measurement, and was less than for *F. oxysporum* (Table 2). These results indicate that *C. fimbriata* produces a pectic acid depolymerase (DP, endopectinase).

Table 1.—Pectin methylesterase activity compared in *Ceratocystis fimbriata* and *Fusarium oxysporum* f. *lycopersici*.

Expt.	Conditions	C. fimbriata	F. oxysporum
Enzyme extract A[a]			
1	still culture for 4 days	−4.25[d]	+11.25[d]
1	still culture for 9 days	−4.50	+ 8.50
2	still culture for 5 days	−1.0	+11.75
Enzyme extract B[b]			
1	still culture for 7 days	−0.25	+29.75
1	Czapek-Dox solution for 7 days		+44.75
2	still culture for 7 days	+0.25	+29.75
2	Czapek-Dox solution for 7 days		+22.0
Enzyme extract C[c]			
1	culture for 5 days	−0.25[e]	+87.0[e]
2	culture for 8 days	−1.75	+15.75

[a] Extract from potato extract medium containing glucose and pectin.

[b] Extract from potato extract medium containing glucose, pectin, casein hydrolyzate and vitamin B_1.

[c] Extract from wheat bran medium.

[d] μeq of acidic groups released (+) or disappeared (−) within 90 minutes.

[e] Reaction time: 150 minutes.

DP activity was appreciable in the infected tissue, though the amount was small, but no DP activity was found in healthy tissue adjacent to the infected tissue (Table 3). The infected tissue was fragile 2–3 days after the inoculation, which may be due to the action of hydrolytic enzymes such as DP produced by the pathogen. Enzyme extracts from infected and healthy tissue, prepared by homogenization with the buffer, showed an intense brown, in contrast to the light brown of the enzyme solutions from the acetone powders. In both cases, however, enzyme activity was about the same and found only in infected-tissue extract.

Cellulase activity.—Wheat bran extract was prepared in a way similar to that for assay of pectolytic enzyme activity, and used for investigation of cellulase activity. Activity in infected tissue and also in adjacent healthy tissue was investigated with the extracts used for assay of DP activity (enzyme extract IV, Table 3). Cellulase activity was found in both wheat bran extract and infected tissue, but not in healthy tissue. Activity in the wheat bran extract was less than that from *F. oxysporum* (Table 4).

Nature of pectolytic enzymes of C. fimbriata.—DP, which splits the glycosidic linkage at random, has been classified into 2 groups (4, 5): one decomposes polypectate much more rapidly than pectin, and the other decomposes pectin rather than polypectate. The 2 groups may be respectively called endopolygalacturonase (endo-PG) and endopolymethylgalacturonase (endo-PMG) (4). Furthermore, it has been observed that depolymerases may vary in pH optimum (3, 5). On the other hand, there has been found an exopectinase that hydrolyzes only the terminal glycoside bond of polypectate or pectin. The results of the viscosity studies have shown that a DP is produced by *C. fimbriata*. The next step was to clarify the kind and nature of the pectolytic enzymes of the pathogen. For the purpose, the pH-activity curves were investigated on 2 substrates, polypectate and pectin. The activity was determined by reduction in viscosity and a release of reducing groups.

The results of reduction in viscosity are discussed first. With polypectate as the substrate, activity was highest at pH 5.8, with no significant activity above pH 7.5 (Fig. 3). With pectin as the substrate, activity was high at 2 pH values, pH 4.7 (peak A) and pH 8.1 (peak B). The assay for production of the reducing groups showed that the pH-activity curves were the same for reducing-group formation as for reduction in viscosity (Fig. 3). It was also found that reduction in viscosity was high, but the formation of the reducing group was relatively low (Table 5).

Table 2.—Polypectate decomposition by reduction in viscosity and reducing-group production at pH 4.5, *Ceratocystis fimbriata* compared with *Fusarium oxysporum*.

Expt. no.	Conditions	Reduction in viscocity (%)[e]		Released reducing groups (μeq)		Incubation time (min)
		C. fimbriata[a]	F. oxysporum	C. fimbriata	F. oxysporum	
Enzyme extract A[a]						
1	for 4 days	11.3	14.8	0.15	0.40	90
1	for 9 days	10.3	14.6	0.10	1.10	90
2	for 5 days	4.3	16.6	0.40	1.70	180
Enzyme extract B[b]						
1	for 7 days	18.1	15.3			
	Czapek-Dox		6.8			
Enzyme extract C[c,d]						
1	for 5 days	63.2	15.6	1.6	2.35	180
2	for 8 days	73.7	22.1	0.7	1.4	120

[a] Extract from potato extract medium containing glucose and pectin.
[b] Extract from potato extract medium containing glucose, pectin, casein hydrolyzate, and vitamin B_1.
[c] Extract from wheat bran medium.
[d] When viscosity was measured at pH 4.5, precipitates appeared during the viscosity measurement; therefore, pH 6.3 was used in place of pH 4.5. In the case of activity of reducing-group production, pH 4.5 was used.
[e] Incubation time: 60 minutes.

Table 3.—Polypectate decomposition by reduction in viscosity with extracts of diseased sweetpotato tissue.

Enzyme extracts[a]	% reduction in viscosity within 60 min.	
	Injured tissue	Healthy tissue
I	12.5	0
II	9.4	0
III	8.3	0
IV	10.0	3.7

[a] I and II: extracted by grinding tissues with the buffer solution.
III and IV: extracted by homogenizing acetone powder of tissues with the buffer solution.

Table 4.—Cellulase activity of *Ceratocystis fimbriata* compared with that of *Fusarium oxysporum* f. *lycopersici*.

Enzyme extract from	% reduction in viscosity at pH 5 by 3 ml of extract within 60 min.	
	C. fimbriata	F. oxysporum
Wheat bran	39.5	95.0[a]
Infected tissue	31.7	
Healthy tissue	0	

[a] Only 0.5 ml of enzyme extract was used.

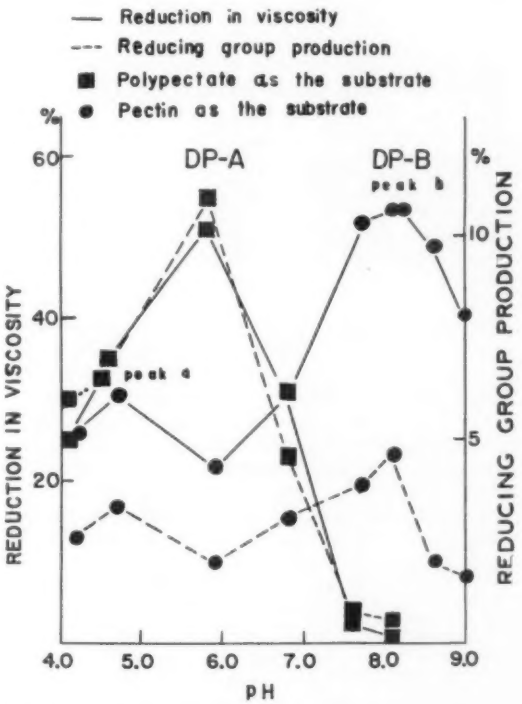

Fig. 3.—pH-activity curves of the pectolytic enzymes.

Reduction in viscosity was 50% within 30 minutes with both polypectate and pectin; in contrast, less than 5% of the reducing groups was released in 3 hours, even though the enzyme solution was concentrated 4 times as much for reducing group studies as for the viscosity study. From these experiments, it has been assumed that the pathogen does not produce appreciable exopectinase, but forms at least 2 kinds of depolymerase. One has an optimum pH at 5.8 and hydrolyzes polypectate more rapidly than pectin; we call it polypectate depolymerase A (DP-A). The other has an optimum pH at 8.1 and acts only on pectin but not polypectate; it is called pectin depolymerase B (DP-B). It was plausible that the depolymerase showing an optimum pH at 5.8 with polypectate was the same as that showing an optimum pH at 4.7 (peak A in Fig. 3) with pectin, but it should be different from the enzyme that decomposes

pectin at maximum rate at pH 8.1 (peak B in Fig. 3). To test this assumption, 5 different enzyme preparations from the wheat bran extracts were tested for reduction in viscosity at pH 5.8 in the presence of polypectate and at 4.7 and 8.1 in the presence of pectin. Percentage reduction was converted to activity units. To convert % reduction at pH 4.7 to units, the curve for DP-B at pH 8.1 was used. The ratios of DP-A (pH 5.8) to DP-B units (pH 8.1) and of DP-A units to units observed at pH 4.7 were calculated. As shown in Table 6, the ratio of A units to B units shows values of 0.53–1.03, but the ratio of A units to pH 4.7 units was more constant (2.46–2.68). These results suggest that DP-A is different from DP-B, but is probably the same as the enzyme that decomposes pectin at maximum rate at pH 4.7.

Table 5.—Reduction in viscosity compared with reducing group production.

Substrates	Final enzyme dilution	Incubation time	% reduction in viscosity	% reducing groups released
Polypectate[a]	1:175	30 min	50	
		2 hr	80	
	1:39.7	3 hr		4.7
		6 hr		11.0
Pectin[b]	1:175	30 min	55.3	
		2 hr	83.2	
	1:39.7	3 hr		3.9
		6 hr		9.8
Pectin[b]	1:175	30 min	41.3	
		2 hr	73.5	
	1:4.7	3 hr		3.1
		6 hr		4.7

[a] pH of reaction mixture: 5.8.
[b] pH of reaction mixture: 8.1.

Table 6.—Ratios of activity of depolymerase-A to that of depolymerase-B and to activity at pH 4.7.

Enzyme extracts[a]	Ratio of activity A/B	Ratio of activity A/pH 4.7
I	0.87	2.64
II	0.91	2.46
III	0.55	2.68
IV	1.01	2.56
V	1.03	

[a] Enzyme preparations from wheat bran medium.

Change in the enzyme activities during storage of the enzyme preparation.—The concentrates of the wheat bran extracts were diluted to a concentration appropriate to the assay, and the diluted solutions were stored at −5°C. During storage, the same solutions were thawed and assayed several times. As shown in Table 7, both DP-A and DP-B showed no noticeable change during storage for 10 days, but activities were decreased some in storage for about 1 month or more.

Effect on depolymerase activities of some metabolites in diseased sweet potato.—When infected by the pathogen, the host produces polyphenols such as chlorogenic and isochlorogenic acids, and coumarins such as umbelliferon and scopoletin in the healthy tissue adjacent to the infected tissue, and terpenoids such as ipomeamarone in the infected tissue (1, 21, 22, 23). Chlorogenic acid (final concentration, 311 mg%) inhibited the activities of both DP-A and DP-B only slightly. The concentration used in the experiment was about the same as that in the diseased sweet

Table 7.—Change in activities of the depolymerases during storage in frozen condition.

Enzyme preparation[a]	Storage periods (days)	Units/ml	% of remained activities
DP-A preparation I[b]	0	1.92	100
	"	1.92	100
	"	17	1.87	97.5
II	0	2.00	100
	"	19	1.86	93.0
	"	35	1.67	83.5
DP-B preparation I[c]	0	2.22	100
	"	48	2.32	104.3
II	0	1.50	100
	"	52	0.98	65.4
III	2	1.47	100
	"	10	1.35	91.8

[a] Varied enzyme extracts from wheat bran extract.
[b] Substrate = polypectate.
[c] Substrate = pectin.

Table 8.—The effect of some metabolites in the diseased sweet potato on depolymerase activity.

Metabolites	Final concentration ($\times 10^{-3}$M)	Final concentration (mg%)	Depolymerase-A[a] Control (units)	Depolymerase-A[a] Metabolite (units)	Depolymerase-A[a] Inhibition (%)	Depolymerase-B[a] Control (units)	Depolymerase-B[a] Metabolite (units)	Depolymerase-B[a] Inhibition (%)
Chlorogenic acid[b]	8.57	311	1.85	1.77	4.3	1.79	1.76	1.4
Chlorogenic acid[e]	10.0	363	1.75	1.65	5.7			
Umbelliferon[c]	2.65	43	1.80	1.91	0	1.53	1.43	6.5
Ipomeamarone[d]	3.45	86.3	2.27	2.26	0	2.19	2.12	3.2

[a] Assay method: as shown in Methods. Each inhibitor was incubated with the substrate and the buffer for 10 minutes, and preincubated enzyme solution added. The units of activities were obtained following the procedures shown in Methods; the mean % reduction at 30 and 60 minutes is reported.
[b] Chlorogenic acid: commercial chlorogenic acid recrystallized twice from water. m.p. 202-205°.
[c] Umbelliferon: synthetic preparation m.p. 231-2°.
[d] Ipomeamarone: prepared by redistillation (120-135°/2.5 mm Hg) from ether extract of sweet potato infected by *C. fimbriata* (1).
[e] One ml enzyme solution, 1.1 ml of the chlorogenic acid solution, and 1.2 ml of the buffer (pH 5.8) were mixed and preincubated 2 hr at 30°C. After that, 3 ml of the above mixture was put into the mixture of 3 ml of the substrate (polypectate) and 1 ml of the buffer (pH 5.8) and the viscosity was measured.

potato, 400 mg% (21, 22). Preincubation of the preparations with chlorogenic acid did not cause much inhibition to DP-A. Umbelliferon (final concentration, 43 mg%) did not inhibit DP-A, but did inhibit DP-B a little. DP-B activity was measured at pH 8.1, and in the alkaline pH, the ester linkage in the molecule of umbelliferon may be opened partly and the open form may show some inhibition. The amounts of umbelliferon in the diseased sweet potato are not clear, but seem to be 50 mg/g or less (23). Ipomeamarone (final concentration, 86.3 mg%) did not inhibit DP-A, but did inhibit DP-B a little. It is produced in the infected tissue in the range of 0.5–2.0% (21, 22). If it could be added to the reaction mixture in the same amounts as in the natural condition, it might inhibit both enzyme activities more. Using larger concentrations was impossible, however, since ipomeamarone's solubility is less than 0.1%. From these data it was concluded that chlorogenic acid, umbelliferon, and ipomeamarone do not significantly inhibit the pectin-splitting enzymes at the above-mentioned concentrations (Table 8).

Inhibition of the depolymerases of sweet potato extract.—In investigating the effect of the oxidized chlorogenic acid on the depolymerases, it was found that sweet potato extract showed inhibitive action to the enzymes. In these experiments, sweet potato extract was prepared as follows: 10 g of the tissue (var. Orange Little, more susceptible, though showing some resistance) were homogenized in 10 ml of distilled water. The homogenate was filtered through cheesecloth and centrifuged 30 minutes at 10,000 rpm, and the supernatant was dialyzed against distilled water for 20 hours. When the dialyzed extract was incubated for 2 hours with the enzyme preparations from bran cultures, both the depolymerase activities were inhibited more than 95% (Table 9). When the extract and the enzyme solution were preincubated together, the inhibition increased with time. Inhibition was less when the preincubation period was omitted. This shows that rate of inactivation is not rapid. Heating the extract in boiling water for 25 minutes destroyed most of the inhibitory action. The inhibitor was precipitated by saturated ammonium sulfate. Since the inhibitor in sweet potato extract is non-dialyzable, heat-labile, and precipitated with ammonium sulfate, it seems to be a high-molecular compound, probably a protein.

Sweet potato extract showed no inhibition of cellulase in the wheat bran extracts on which the pathogen was grown. To determine whether the inhibition was specific to the enzyme of the pathogen, other depolymerase preparations such as pectinol (a commercial depolymerase) and a concentrated cultural medium of *F. oxysporum* f. *cubense* were tested for inhibition by the sweet potato extract. As shown in Table 10, the extract showed 80–85% inhibition of DP-A, but only about 20–30% inhibition of the commercial or *Fusarium* depolymerases. Furthermore, in contrast to the result on the enzyme solution of the pathogen, no gradual increase in inhibition with time was found with the latter depolymerases. Therefore, this inhibition seems to be more specific to the depolymerases of *C. fimbriata* than to other depolymerases and suggests that the sweet potato contains a substance, possibly an enzyme, that is capable of specifically inactivating the pectolytic enzymes of the sweet potato pathogen.

Table 9.—The effect of sweet potato extract on depolymerase activities.

Depolymerase A			Depolymerase B		
Control[a]	Extract[b]		Control[a]	Extract[b]	
Units	Units	Inhibition (%)	Units	Units	Inhibition (%)
1.77	0.06	96.5	1.39	0.07	95.0

[a] Control: equal volumes of the enzyme solution (wheat bran extract), distilled water, and buffer (pH 5.8) were mixed and preincubated 2 hr at 30°C. The assay procedure was as described in Methods.

[b] Extract: sweet potato extract was substituted for distilled water.

Discussion.—It was found that *C. fimbriata* produced 2 pectolytic depolymerases but not pectin methylesterase. When compared to *F. oxysporum* f. *lycopersici*, polypectate depolymerase was produced almost at the same level on some cultural media except for finding a larger activity when grown on wheat bran. The pathogen showed less depolymerase activity on injured sweet potato tissue than did *F. oxysporum* f. *lycopersici* on tomato stem sections (3) and *R. stolonifer* on sweet potato tissue (19). This may be explained by both the production of less

Table 10.—The effect of sweet potato extract on activities of other depolymerase preparations.

Source of enzyme preparation		Reduction in viscosity % within 30 min[a]	Units	Inhibition %	Reduction in viscosity % within 30 min[a]	Units	Inhibition %
Ceratocystis fimbriata	control	27.2	0.72		41.6	0.72	
	extract	6.9	0.14	80.5	8.1	0.10	86.2
Pectinol	control	46.5	1.66		63.3	1.73	
(10,000 dilution)	extract	40.0	1.30	21.7	55.6	1.27	26.6
Fusarium oxysporum f.	control	32.2	0.91		47.8	0.91	
cubense	extract	28.6	0.76	16.5	43.6	0.77	16.5

[a] The reaction mixtures contained the substrate (3 ml), buffer (1 ml), sweet potato extract concentrated by ammonium sulfate (1 ml), distilled water (1 ml), and enzyme solution (1 ml). The control contained distilled water in place of the extract.

enzyme and the action of an inhibitive factor, probably a protein in healthy sweet potato, or by inhibition by some metabolites in the diseased sweet potato.

Metabolites as chlorogenic acid, umbelliferon, and ipomeamarone did not inhibit the depolymerases, but other metabolites such as the oxidized substances from the polyphenols produced in the injured part, may cause an inhibition. Grossmann (11) and Harthway and Seaking (12) reported that simple tannin substances, such as chlorogenic acid, did not inhibit pectolytic enzymes, but that condensed tannins, such as cryobalans tannin, showed some inhibitive effect. Wood's group (2, 29) found an inhibitive action of oxidized substance of leuco-anthocyanin on the enzyme of *Sclerotinia fructigena*, but no inhibition with oxidized chlorogenic acid.

Weurman (26) found inhibitors to pectolytic enzyme from pear, which seem to be high molecular compounds. Echandi and Walker (7) found factors reducing the maceration activity of the pectolytic enzyme of *S. sclerotiorum* in extracts of some stored organs. The pectolytic enzyme inhibitor found in sweet potato tissue may be similar to these. It seems to be a protein that strongly inhibits activity of both depolymerases of *C. fimbriata* but does not reduce the cellulase activity of the pathogen. The inhibitor was much less active toward pectolytic enzymes from the other fungi, and the mode of action was different from that on the depolymerases of the pathogen. It is interesting that it requires time for complete inhibition. This suggests that the depolymerases may be inactivated by an enzymatic action of the inhibitor or by a slow combination of the inhibitor with the depolymerases. Another possibility might be a slow combination of the enzyme and a complex produced by reaction of that factor in sweet potato with other substances in bran extraction. To clarify the mechanism of inhibition, other experiments should be added, such as tests on more purified enzyme preparations from bran and other culture media as well as concentration of the inhibitory factor. The physiological meaning of this factor in sweet potatoes may be clarified after we understand the inhibitory mechanism in more detail. It is of special interest because of its much greater action toward pectolytic enzymes from the fungus that attacks sweet potato.

How are the pectic substances in the cell wall broken by the hydrolytic enzymes of the pathogen? DP-A and DP-B respectively decompose pectin of low and high methoxy content, hence almost all pectin substances in the cell walls may be decomposed by these two enzymes. The role of the cellulase of the pathogen that decomposes carboxymethyl cellulose will not be clear until a series of studies is carried out including the other kinds of cellulases, such as C_1 (16).

What is the function of these hydrolytic enzymes, especially depolymerases, with regard to pathogenicity? There are several possibilities:

1) The degradation of pectic substances to provide nutrients and energy for growth and penetration of the pathogen. In sweet potato roots, sucrose and starch are respectively about 4–5% and 20%. In contrast, pectin is present in the cell wall in an insoluble form, and its amount is much less, probably 4–5% as calcium pectate in the cell wall materials (13, 20). The depolymerase enzymes of the pathogen do not produce appreciable amounts of the monomer, galacturonic acid. Therefore, the contribution of the depolymerase-decomposed pectic substances as nutrients to the fungal growth in the host tissue should be small.

2) Maceration of host cells to allow the mycelium to penetrate the killed cells and absorb nutrients. This occurs with soft rots but not with dry rots, such as black rot of sweet potato, because the pathogen does not produce sufficient pectolytic enzymes to macerate the cells at the first stage of infection. It has been often observed, however, that the susceptible tissue infected becomes more fragile in later infection. This may be caused by the action of such cell-wall-decomposing enzymes.

3) Decomposition of the cell wall of the host to help penetration. Anatomical observations showed that mycelia penetrate not only along the outer part of the cell wall but also into the cells. It is possibe that at the stage of the penetration into the cells, some hydrolytic enzymes such as depolymerases are produced at the limited part where the mycelia penetrate, making penetration easier. To confirm this assumption, the enzyme activities in the cells just infected by the pathogen should be measured on a micro-scale.

4) Decomposition of pectin substances to disturb active metabolism related to instant necrosis or to the hypersensitive reaction. When the tissue of the resistant variety is attacked by the pathogen, instant necrosis (denaturation, coagulation of proteins, ipomeamarone formation, and browning) precedes penetration of the pathogen into the healthy tissue. Instant necrosis (hypersensitivity) accompanying the respiratory increase plays an important role in the defense mechanism against the pathogen in resistant varieties by producing antipathogenic agents and forming protecting barriers (21, 22). The tissue of the susceptible variety shows some metabolic alterations similar to those in the resistant variety, but not as strong. The pathogens continue to penetrate the tissue, where the brown reaction is not yet observed, and such infected cells are not killed rapidly; thus, no sudden necrosis is shown. Ipomeamarone formation and, sometimes, brown reaction are seen, though weak. Pectin depolymerases may be produced at that stage in such a small amount that maceration death may not occur, but some active metabolism related to defense action may be disturbed. Ipomeamarone may be formed in a small amount through a slight injury of cell wall metabolism by the enzymes, and it would accelerate metabolic inhibition (21, 22). We have observed that both pectinol solution (a commercial crude pectinase preparation) and the concentrated cultural media of the pathogen induce ipomeamarone formation, but it has not yet been clarified

whether pectolytic enzymes are factors important to its formation in susceptible infected tissues.—Department of Biochemistry, University of Wisconsin, Madison.

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BOOK LISTINGS

Maladies a virus des plantes cultivées et méthodes de lutte.—*Cornuet.* P. 1959. Institut National de la Recherche Agronomique, Paris. 440 p., 42 plates.

This is the first complete textbook in French on viral diseases of plants. Diseases occurring in France or in nearby countries are described in full, by crops. Literature citations are given throughout the text. Several supplements include: physical properties, principal host plants, and insect vectors for causative viruses; similar data for viruses not known to occur in or near France; viruses transmissible through seeds of their hosts; soil-transmitted viruses; a classification of plant viruses according to method of dissemination; and a list of viruses of wide host range. The volume is provided with an index to plant hosts as well as a detailed table of contents.

Illustrated genera of imperfect fungi. *Barnett, H. L.* 1960. 2nd ed. Burgess Publishing Co., Minneapolis, Minnesota. 225 p. $4.50.

The second edition is organized in the same manner as the first, but the number of genera illustrated (462) is about 46% greater. Thirty-three pages are devoted to a key to the included genera, 168 pages to descriptions and illustrations, 16 pages to references, and 5 pages to the index.

PARASITIC AGGRESSIVENESS IN PHYTOPHTHORA INFESTANS

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SUMMARY

Two factors related to parasitic aggressiveness (inoculum concentration and speed of infection) were studied for several isolates of *Phytophthora infestans*. The ratio of infection to concentration of sporangia in the inoculum suspension was fairly constant over a range of sporangial concentrations of about 1200-12,000/ml, and did not differ greatly between isolates. More than 12,000/ml tended to obscure differences between isolates, less than 1200/ml apparently increased sampling error. In potato plants inoculated with sporangia in the greenhouse at 20°C, infection was first appreciable about 4 hours after inoculation. Thereafter, the number of infections per plant increased with time, although after 22 hours some isolates produced as much as six times as many lesions as others. Such differences were found between isolates of the same or different pathogenic races; also the speed of infection was not necessarily a function of speed of sporangial germination.

Introduction.—The term parasitic aggressiveness as used in this paper refers to the ability of a pathogen to increase to epidemic proportions in a host population. A number of characteristics probably contribute to aggressiveness in plant-pathogenic fungi. It is partially dependent upon the ability to produce large amounts of viable inoculum that can be effectively disseminated. The speed with which a fungus can penetrate and infect the host can also contribute to aggressiveness.

Breeding of potato (*Solanum tuberosum* L.) varieties that are "field resistant" to late blight is a problem directly affected by aggressiveness. As used in this paper "field resistance" refers to the sum of all those plant characteristics that tend to limit the spread of a disease in a population of plants. Although the nature of field resistance to late blight is not well known, it is reported to be influenced by such factors as delayed penetration, slow invasion of host tissue after penetration, reduced sporulation, and lack of stem lesions (1, 4, 9, 10, 12).

Although field resistance appears not to be affected by the different races of *Phytophthora infestans* (Mont.) d By. as determined by pathogenicity on the differential varieties described by Black et al (2), there is a possibility that highly aggressive races could appear and be perpetuated on field-resistant clones. Toxopeus (16) reported that the increase of late blight incidence on the variety Voran in The Netherlands (over an 8-year period in which acreage increased considerably) is due to the appearance of a race of *P. infestans* that became adapted to the variety. Niederhauser and Cervantes (11), on the other hand, reported that field-resistant clones did not appear to become more susceptible over a 5-year period of field testing in Mexico.

Differences among isolates that might affect aggressiveness have been reported by several workers. Castronovo (3) found differences between isolates of *P. infestans* in the speed with which they infect potatoes and in the number of lesions resulting from inoculation with similar numbers of sporangia. Although Crosier (5) reported that the temperature ranges for sporangial production by the isolates he used were 3–26°C in a saturated atmosphere, Wallin (17) found sporangia on plants in the field when the temperature was about 30°C and the relative humidity 40–50%. Larance and Martin (7) and Martin (8) found differences in the ability of isolates to survive high temperatures. A range of survival times from 6 hours to 6 days was found among isolates maintained at 36°C. Thomas (13) reported differences in the survival ability of sporangia among isolates of *P. infestans* from Colorado and Maine. Wallin and Hoyman (18) demonstrated the 5 isolates of *P. infestans* differed in ability to survive high post-inoculation air temperatures.

The following study was undertaken to get information on the range of aggressiveness in *P. infestans*. The effect of inoculum concentration on infection and the relative speed with which different isolates penetrate and infect the host were considered.

Materials and methods.—All late blight cultures used in this study were originally isolated from infected potatoes grown in Minnesota. The pathogenic specificity of the isolates was determined by inoculating differential potato varieties (2).

The cultures were maintained and inoculum prepared as described by Thurston (14). Occasionally sporangia were obtained from infected sporulating leaves of Cobbler potato plants. Either the entire plant was placed in a moist chamber or the infected leaves were detached and placed in a moist atmosphere where sporangia were produced.

A sporangial suspension was made by washing the mycelia of the cultures or plant leaves with distilled water and, after thorough mixing, counts were made with a haemocytometer on 4 samples from each lot. The suspensions were then diluted with distilled water to the desired concentration. The suspensions were kept in an ice-water bath during the counting and dilution procedure. A sample of each sporangial suspension was placed at 10°C at the time they were made, and percentages of germination determined by counting after 18–20 hours (Fig.1).

Inoculum was applied with a hand sprayer attached to an air pump adjusted to deliver air pressure at 2 lb/sq in. Cobbler potato plants 12–15 in. tall were

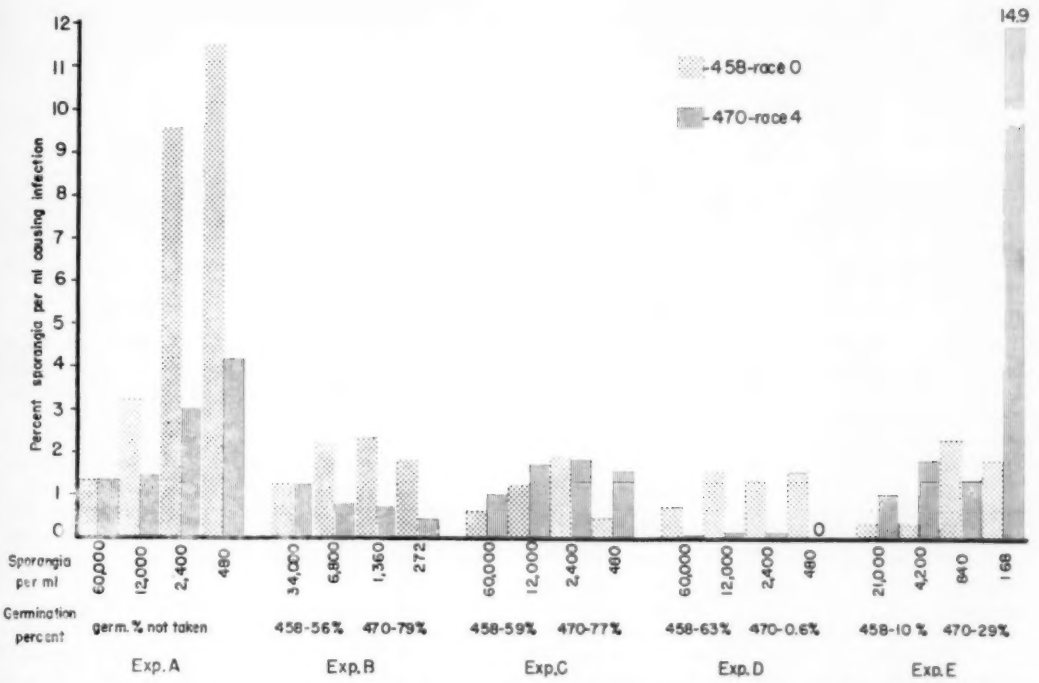

Fig. 1.—The effect on percentage infection of potato leaves of 4 concentrations of sporangia from isolates 458 and 470 of *Phytophthora infestans*.

wet with water before inoculation. The inoculum was applied to the underside of the leaves, each plant being sprayed for 8 seconds. The plants were placed immediately in moist chambers. In studies on the speed of infection, plants were placed on a turntable that rotated about 60 rpm while the inoculum spray was directed at the undersides of the leaves. Each plant was sprayed for 7 seconds.

The inoculated plants, in studies on the concentration of inoculum, were incubated 12–24 hours in moist chambers consisting of metal boxes of 20 × 20 × 20 in. Relative humidity (RH) was maintained at 100% by putting about 1 in. of water in the bottom of the metal box and draping wet cloth sacks over the top before placing the cover in position. The inoculated plants in the studies on speed of infection were incubated in a moist chamber consisting of a 42 × 32 × 60-in. wooden frame over which polyethylene plastic was stretched. A mechanical humidifier was used to maintain 100% RH in the chamber.

Four to 8 hours after the first symptoms of late blight were observed, the number of lesions were counted on the terminal and the first two lateral leaflets on 6 leaves of each plant. At this stage the lesions were small and could be easily counted.

Results.—*Effect of concentration of inoculum on infection by different isolates.*—To compare the infectivity of 2 isolates of *P. infestans*, sporangia of isolate 458 (race 0) and 470 (race 4) were produced on either sterilized yellow field peas (*Pisum arvense* L.) or Cobbler potato plants. The original sporangial suspensions were diluted 1:5, 1:25, 1:125, and 1:625; suspensions of both isolates were equal in any given experiment although the undiluted preparations varied from 21,000 to 60,000 sporangia per ml in different experiments. The suspensions were sprayed on Cobbler plants; 1 plant sprayed with each concentration of each race was incubated 12–24 hours in the same moist chamber. There were 5–8 replications of each test.

The mean number of lesions caused by each isolate in 5 different tests appears in Table 1. Tests A, B, and C were analyzed for statistical significance; tests D and E were not analyzed, because the differences were large and obviously due to differences in percentage germination. (See Fig. 1.) The data for the 1:625 dilution were omitted because they varied widely and the samples were obviously inadequate. In Fig. 1 the same data are converted to show the percentages of sporangia per ml that caused infection. This, of course, does not tell what actual percentages of the sporangia are capable of causing infection, since many of them never fell on the plants during inoculation. They are comparable, however, because each plant was inoculated in the same manner.

Isolate 458 tended to cause more infection than isolate 470 in a majority of the comparisons; there were exceptions, however, e.g., experiments C and E.

Table 1.—Effect of concentration of inoculum of 2 different isolates of *Phytophthora infestans* on actual mean lesions per potato plant.[a]

	Experiment A		Experiment B		Experiment C		Experiment D		Experiment E	
Dilution	458	470	458	470	458	470	458	470	458	470
1:0	769	778 NS[b]	399	474 NS	364	584 *	412	16	70	216
1:5	381	282 NS	150	60 *	144	204 NS	184	12	12	77
1:25	227	72 **	33	10 *	26	44 NS	31	3	19	11
1:125	51	14 **	5	2	2	7 NS	7	0	1	5

[a] Mean lesions per plant here refers to the total number of lesions on the terminal and first two lateral leaflets of 6 leaves on a plant. 458 and 470 are 2 isolates of *P. infestans*.
[b] NS, no significant differences between isolates; *, significant at 5% level; **, significant at 1% level. Data from experiments D and E were not analyzed.

There was a tendency for percentage of infection to increase with a decrease in concentration from 1:0 to 1:5, and then to level off with further dilution. Experiment A is an exception, but the trend is present in experiments B and C and for isolate 458 in experiment D.

In Table 2 the data were calculated to show the percentage of sporangia infecting, assuming 100% germination. This was done by dividing the percentages shown in Fig. 1 by the germination percentages. Under the conditions of this experiment about 1–4% of the sporangia that germinate caused infection if the actual amount of germination was at least 50%. If 29% or less of the sporangia germinated, from 0 to 37% appeared to infect. Obviously, comparisons of races with respect to infectibility should not be made unless the percentage of sporangial germination exceeds 29%.

The cause of the differences in germination and the erratic infection associated with this is not known. A limited comparison of inoculum from pea cultures and from Cobbler plants indicates no difference between sporangia from the two sources in ability to cause infection (see Table 2).

These experiments show in general that a fairly uniform percentage of infection can be obtained at sporangial concentrations of 1200–12,000/ml. Above and below these concentrations, limitations are encountered; at 12,000 and above, space on the host surface may be limiting, and at 1200 and below, sampling was probably inadequate, especially when germination was poor. Another source of error may be in the application of inoculum to leaves.

Speed of infection.—The time necessary for *P. infestans* to infect was studied by Crosier (5), who found that swarmspores would infect in 2 hours at 21°C and 100% RH. The number of penetrations declined at temperatures above and below 21°C, but even at 10°C the fungus could establish itself in 2½ hours.

To determine whether isolates of *P. infestans* differed in speed of infection, several pairs of isolates were compared. Sporangia were obtained from cultures grown on sterilized peas or from infected Cobbler potato plants and suspensions were made with 8000–30,000 sporangia per ml.

The plants were inoculated and incubated as described in Materials and methods. At intervals of 2, 3, 8, and 22–24 hours, 4 plants inoculated with each isolate were removed from the moist chambers and dried with an electric fan to stop further germination and penetration by the fungus. Portions of the sporangial suspension were incubated in the same moist chamber, and samples were taken for germination counts whenever plants were removed. Four to 8 hours after symptoms appeared, the lesions were counted on 6 leaves of each plant. The results are shown in Fig. 2. Statistically significant differences

Table 2.—Effect of concentration of inoculum of 2 different isolates of *Phytophthora infestans* on the percentage of sporangia per ml that infect, assuming 100% germination.

	Per cent sporangia infecting (100 per cent germination basis)							
	Experiment B 34 000/ml[b]		Experiment C 60,000/ml		Experiment D 60,000		Experiment E 21,000	
Dilution	458	470	458	470	458	470	458	470
1:0	2.09	1.76 NS[a]	1.03	1.26 NS	1.09	4.44[a]	3.44	3.55[a]
1:5	3.94	1.12 *	2.03	2.22 NS	2.43	16.71	2.95	6.31
1:25	4.26	0.96 *	1.83	2.38 NS	2.04	20.83	23.33	4.52
1:125	3.09	0.74 *	0.54	1.85 NS	2.29	0.0	36.90	10.24
Germination % of sporangia	56	79	59	77	63	0.6	9.7	29
Source of inoculum	pea culture		Cobbler plants		Cobbler plants		pea culture	

[a] NS, no significant differences between isolates; *, significant at 5% level; **, significant at 1% level. Data from experiments D and E were not analyzed.
[b] Original concentration; sporangia per ml.

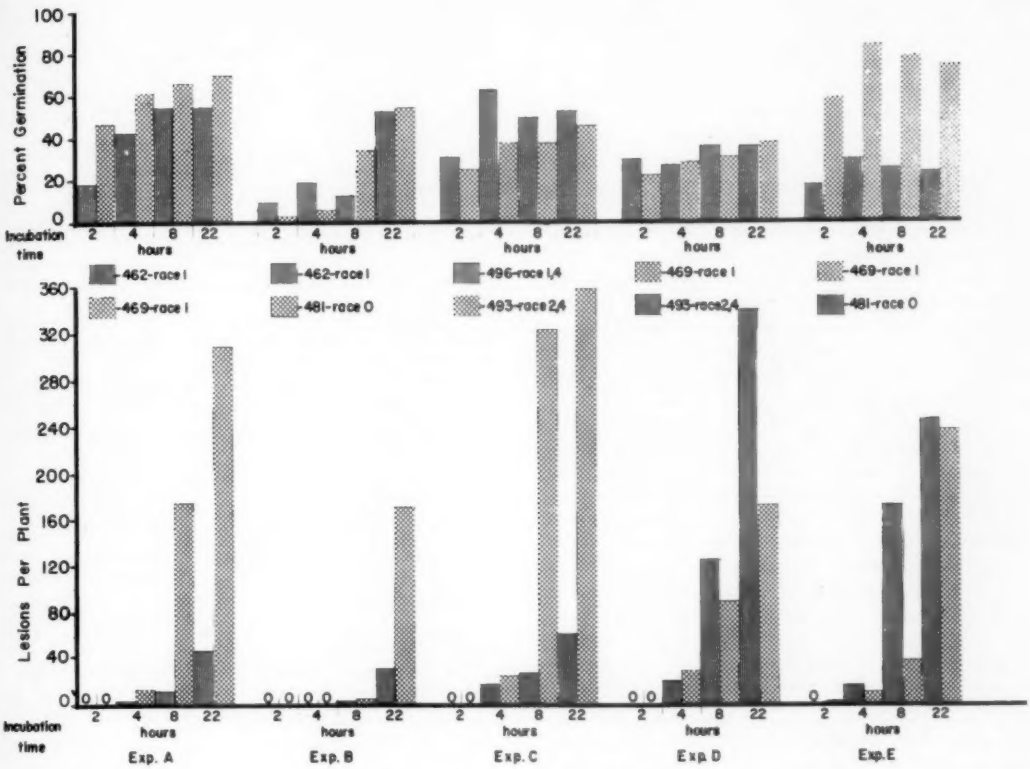

Fig. 2.—Speed of penetration and infection of 5 isolates of *Phytophthora infestans* as related to sporangial germination.

between isolates were found in each test except experiment E.

In two comparisons, the differences between the isolates were considerable by 8 hours (Fig. 2-A,C), whereas in Fig. 2-B,D they were not great until 22 hours. Differences present at 8 hours are probably important in evaluating the aggressiveness of any given isolate because 8-hour periods of weather favorable for infection are more likely than a 22-hour period.

The relation between germination rate and infection was not consistent; the differences between compared races were too small to indicate a correlation between germination rate and infection. In fact, in experiment E, isolate 469 germinated faster than 481 but caused less infection.

No appreciable infection occurred until 4 hours of incubation. Crosier (5) found that 2 hours was sufficient, but he inoculated with zoospores instead of sporangia.

Discussion.—These experiments show that it is possible to demonstrate differences in infectiveness between isolates of *P. infestans*. The importance of this fact is threefold: first, information about the precision of infection tests will be useful in improving techniques for testing for field resistance; second, knowledge of the diversity in aggressiveness of the pathogen will aid testing of new clones and possibly provide some basis for predicting the stability of their resistance; and third, this work contributes to an understanding of epidemiology of late blight.

Field resistance in the host, like aggressiveness in the pathogen, is measured principally by the number of infections that result from a given amount of inoculum. To measure such resistance each plant tested should receive an equal amount of inoculum. It is true that only a small percentage of spores produced by a fungus ever reach an infection court and result in infection and there is no assurance that the ratio of spores to infection is always constant. At very low concentrations a point called numerical threshold of infection (6) may be reached where the chances for escaping infection are great. The commonly used dilution endpoint in the study of viruses is an example of this principle and is here shown in Table 1.

At high concentrations the competition for infection sites would increase so that a relatively low percentage of spores could penetrate. Also, it is conceivable that

at certain concentrations the germinating spores have stimulatory or inhibitory effects on one another. Thurston (15) demonstrated that filtrates obtained from suspensions of germinating sporangia of *P. infestans* reduced the indirect germination of the same isolate and isolates of other races.

With the inoculation technique used in the present studies sporangial concentrations of 1200–12,000/ml were found to result in a fairly consistent percentage of infection. This was equivalent to about 1–10 lesions per leaflet in these studies. Above 12,000 sporangia per ml, differences between isolates in per cent infection are not apparent. Below 1200/ml, the sampling error becomes great, especially if fewer than 50% of the sporangia germinate. No definite relation could be found between optimum inoculum concentration and isolate.

Wide differences were found between isolates in speed of infection (Fig. 2). Differences between isolates of the same pathogenic race were just as great as differences between isolates of different races. This supports Castronovo's (3) contention that race designation in *P. infestans*, which is based on pathogenicity on a set of differential potato clones, does not indicate a grouping of certain other physiological characters.—University of Minnesota, St. Paul.

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SOME OBSERVATIONS ON HYPERSENSITIVITY TO PLANT VIRUSES

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SUMMARY

Grafts made between healthy clones of *Dianthus barbatus* normally hypersensitive to carnation mosaic virus (CMV) and other clones systemically infected with CMV resulted in complete systemic invasion of the hypersensitive clones. Similar results were obtained in grafts involving healthy hypersensitive plants of *Nicotiana glutinosa* and *N. tabacum* Connecticut Havana 423, respectively with *N. tabacum* var. Haronova systemically infected with tobacco mosaic virus. These results, and those of others, indicate that systemic invasion results whenever a continuously high level of inoculum is applied to a hypersensitive host for an extended period of time.

Introduction.—Since Holmes' discovery, in 1929 (6), that the number of necrotic local lesions induced by a virus in a hypersensitive host is related to concentration of the virus inoculum, numerous studies have been made of the virus-host relationships that give rise to a sharply demarcated hypersensitive reaction. In spite of this, very little information has been obtained by experimental means about the mechanisms involved in the localization of virus characteristic of hypersensitive reactions.

Several reports have shown that it is possible to change the virus-host relationship from a local-lesion reaction to a systemic invasion, by specific experi-

mental manipulation. Thus, plants of *Nicotiana glutinosa* L. inoculated with tobacco mosaic virus (TMV) became systemically infected at 35°C or higher (11). Also, in leaves of pinto bean infected with the bean rust fungus, TMV induced necrotic rings not sharply delimited as were the lesions induced in healthy leaves; instead they enlarged at a rate of about 1 mm per day throughout the rusted tissues (14). Since the rust resulted in an increase of the number and amount of free amino acids in bean leaves, Yarwood suggested that this may have had some bearing on the results.

Hutton and Bald (7) described a potato hybrid which when mechanically sap-inoculated with potato virus Y produced only local necrotic lesions. When this hybrid was grafted to an Epicure scion containing virus Y, the hybrid reacted with severe top necrosis in 14 days. This top necrotic reaction appears to be well-established in the literature dealing with potato viruses (3, 9, 10), and was undoubtedly observed quite early, since a combination of sap inoculation and grafting has been used for a long time (8).

A similar result was described (2, 5) when immune hypersensitive varieties of bean were grafted to bean varieties susceptible to bean viruses 1 and 4. The immune and hypersensitive varieties produced necrosis and died when grafted to susceptible, infected varieties. This might perhaps have been expected since Grogan and Walker note (5) that occasional plants of the resistant variety, Idaho Refugee, developed systemic necrosis upon mechanical inoculation with bean virus 1.

These results with bean varieties prompted Walker to state (12) that genetic resistance "does not prevent virus increase and the hypersensitive reaction in the phloem when a continous supply of virus is provided by the approach-graft technique." If this is the case, then it might be possible to induce similar results in other host-virus systems, even when the hosts do not occasionally produce systemic symptoms with mechanical inoculation, as in the Idaho Refugee–bean virus 1 combination. The following experiments show this to be the case.

Methods and results.—The first experiments were with plants of 3 seedling clones of *Dianthus barbatus* L., 2 of which respond to inoculation with carnation mosaic virus (CMV) by the production of brown necrotic local lesions only with no subsequent systemic infection (4). These 2 clones, previously called Clones 20 and 26 (4), will be referred to here as LL20 and LL26. The third clone, S21, previously called Clone 21, developed only systemic symptoms some 10 days after inoculation with CMV, the symptoms consisting of a pronounced mottle, severe twisting of the leaves and a general dwarfing of habit.

Side grafts were made between the 2 types of clones, one of which (the stock) was systemically infected with CMV. Four weeks after the grafts were established both members of the graft were tested for the presence of CMV by recovery tests on plants of clones LL20, LL26, and S21. After sampling, both members of the graft were tested for protection against CMV.

Nine grafts between healthy S21 plants and S21 plants systemically infected with CMV resulted in systemic invasion of the initially healthy scions. The symptoms were similar on the receiving and donor members of the grafts, and CMV was recovered without difficulty from all parts of the graft combination. Reinoculation with CMV had no additional effect. Although organic union was not established in 3 of the 9 grafts, the contact period was still sufficiently long for the virus to be transmitted from the diseased S21 to the healthy S21.

Eight healthy LL20 and 8 healthy LL26 plants were grafted to 16 diseased S21 plants. This resulted in systemic invasion of the LL20 and LL26 members of the grafts. The symptoms on the hypersensitive clones consisted in most cases of long brown necrotic lesions running lengthwise on the leaf; in some cases, a general mottling was induced, and in several plants the growing tip was killed. CMV was recovered readily from any portion of both members of the graft. The systemically infected LL plants appeared to be protected from further inoculation, since inoculation with CMV produced no further symptoms.

Grafts were then made involving *N. tabacum* L. var. Haronova (referred to as Haronova), which becomes systemically invaded by TMV, with *N. glutinosa*, which produces only necrotic local lesions when mechanically inoculated with TMV, and with *N. tabacum* Connecticut Havana 423 (referred to as Conn. 423), which carries the N gene from *N. glutinosa* and thus produces only necrotic local lesions when mechanically inoculated with TMV.

Three young plants of *N. glutinosa* were top or side grafted to large Haronova plants that had been infected systemically with TMV for about 2 months. At the same time, 4 young Conn. 423 plants were grafted in the same way to other large diseased Haronova plants. In addition, 3 mature individual leaves of *N. glutinosa* and 3 mature leaves of Conn. 423 were side-grafted to Haronova plants.

Fourteen to 20 days later, dark brown necrotic spots began to appear on the leaves of *N. glutinosa*, particularly along the veins of young leaves. The lesions on the tip leaves became larger in the next 2 weeks, until finally 2 of the 3 growing points were killed (Fig. 1). The other leaves continued to produce a few necrotic spots, and a slight chlorotic mottle was observed. When these plants were tested for the presence of TMV on *Chenopodium amaranticolor* L. and on Conn. 423, the virus was found to be present in 2 of the 3 plants but in very low concentrations: e.g., inoculum from the necrotic areas induced only 4–5 lesions per leaf, compared to 50 or more lesions induced by inoculum from about the same amount of tissue taken from the donor Haronova plant. Comparable inoculum from mottled areas of leaves of *N. glutinosa* induced 10–15 lesions per assay leaf. Similar results were produced when Conn. 423 was used as the assay plant.

Fig. 1.—Plant of *Nicotiana glutinosa*, about 6 weeks after being grafted to a TMV-infected plant of *N. tabacum* var. Haronova. Note the dead growing point, the spreading dark-brown necrosis, and the almost total absence of other symptoms on the older leaves, from which TMV can be recovered. A leaf of the TMV-infected stock Haronova plant is in the background.

The 3 mature grafted leaves of *N. glutinosa* showed no symptoms except for a few necrotic spots on one leaf, from which TMV could be recovered. All 3 of these leaves became yellow and quite wilted within a short time.

Although organic union formed in none of the grafts between *N. glutinosa* and Haronova, only one graft could be separated easily, some force being required to separate the others. The 3 grafted leaves were easily separated from the stock.

The Conn. 423 plants grafted to diseased Haronova also developed symptoms, but they were considerably milder than those in *N. glutinosa*. Only a few minute lesions developed in the growing tip leaves. Older leaves developed a very dark-green mottle that became more accentuated after 3–4 weeks. The intercostal areas of these leaves also tended to become puckered or shirred.

All 4 of these Conn. 423 plants contained TMV, which could be isolated from either younger or older leaves. The concentration was also low compared to that in the stock Haronova plants, but somewhat higher than in *N. glutinosa*. The 3 mature individual leaves of Conn. 423 did not develop symptoms, but TMV was readily isolated from 1 of the 3 leaves. Two of the 4 grafts had formed organic union when the raffia was removed; the other 2 were separated with difficulty probably because they were in the process of forming a union.

Of the 3 mature leaves that had been side-grafted, 2 formed organic union with the stock Haronova plant; one leaf yielded TMV upon indexing. The second leaf that had formed organic union was re-indexed, but no virus was isolated from it. The third leaf dropped off when the raffia was removed.

Epidermal strippings were made from the grafted Conn. 423 plants and examined microsopically for the presence of inclusion bodies both without staining and after staining with 0.05% trypan blue. As controls, strippings from leaves of healthy Conn. 423 plants of the same age, and from leaves of the TMV-infected stock Haronova plants, were examined. Inclusion bodies of the amoeboid type were readily found in the strippings from the Conn. 423 portions of the grafts. These were rather coarse in appearance and stained deeply blue with trypan blue. They were more easily found in the hair cells than elsewhere. No crystalline inclusions were detected. The strippings from the TMV-infected Haronova plants showed a great abundance of amoeboid inclusions, far greater in number than were found in the Conn. 423 grafts. No such bodies could be found in the healthy Conn. 423 strippings.

Discussion.—In considering the mechanism of hypersensitivity in which the virus remains localized, one can postulate either that some physical or chemical barrier is set up as a result of infection and prevents the virus from spreading beyond the limits of the lesion; or that an equilibrium situation takes place, in which the virus is destroyed as quickly as it is synthesized, perhaps eventually more quickly, so that finally there is a net decrease in the concentration of virus in the lesion; or that both these mechanisms operate simultaneously or consecutively.

In order to overcome such a delimiting situation, it would be necessary for the virus to multiply at a rate increased sufficiently to prevent formation of the barrier or to penetrate such a barrier, and to exceed the rate of destruction of newly synthesized virus within the lesion cells. The alternative would be a continuous supply of virus from an outside source of high inoculum that would provide sufficient virus to accomplish the same result. The results described here, and previous work, are in good accord with this necessary condition. Yarwood's findings (14) suggest that localized nutritional effects may produce an increase in virus multiplication and a consequent increase in invasiveness, although in this case, the spread of virus could be the result of a decrease in the resistance of these cells to virus invasion. Systemic invasion of *N. glutinosa* by TMV at high temperatures (11) could well be the result of increases in the multiplication rate of the virus. Indeed, evidence that

this increase in rate occurs was found in studies on the respiration of such tissues at normal and increased temperatures (13).

The results of grafting systemically infected plants to resistant or hypersensitive plants, involving both potato varieties (3, 7, 9, 10) and bean varieties (2, 5), and the results presented here indicate that, as Walker suggested (12), when a continuously high inoculum enters the hypersensitive host, then the local lesion system can become systemic. This situation produces in effect the same result as an increased rate of multiplication, since sufficient virus continues to enter the hypersensitive host to overcome the localizing mechanisms.

Grafting of this type does not always produce the same extent of systemic invasion. Benson and Hooker (1) have described a graft between plants susceptible to potato virus X (PVX) and 2 potato varieties immune to PVX. Under their conditions, starch usually accumulated above the graft union in the infected susceptible host only. However, if PVX inoculation of the susceptible portion was delayed, then starch did accumulate to some extent also below the graft union in both of the immune potato varieties. The grafts involving potatoes and bean plants (2, 3, 5, 7, 9, 10) produced primarily necrosis and death, while the grafts described here produced not only necrosis, but a more complete systemic invasion of the hypersensitive host, including the development of inclusion bodies. The graft method of inoculating is, of course, different from the inoculation method used by Yarwood (14) and Samuel (11), who introduced the virus through the epidermis by mechanical inoculation. In the various graft experiments, the virus was probably introduced directly into the vascular system, where, potentially at least, it could become immediately systemic. In this connection, the rapid killing of the growing points could be considered an extensive hypersensitive reaction. However, the continuing arrival of inoculum from the donor plant could also result in spread of virus into older, non-vascular tissues without extensive necrosis.

It is not clear why systemically invaded plants of *D. barbatus* were more extensively infected and contained more recoverable virus than did systemically infected plants of *N. glutinosa*, and that Conn. 423 was intermediate in this respect. These differences may have been due to differences in the readiness with which union occurred, although the lack of organic union in 2 of the Conn. 423 plants did not prevent their systemic invasion by TMV, nor did the formation of union in the other 2 Conn. 423 plants increase markedly the amount of TMV that could be isolated. It is possible that other factors were operative. Perhaps the fact that *D. barbatus* has parallel venation and the *Nicotiana* spp. net venation may be a factor, since the virus entered through the vascular system.

Another obvious question raised concerns the source and nature of the virus that systemically invaded the hypersensitive hosts. Is all of this virus derived directly from the donor plants, or does the virus multiply in the hypersensitive host? Is the virus that passes continuously from the donor, or in the case of Yarwood and Samuel's experiments, that multiplies at an increased rate, of the same nature as the virus that is normally unable to overcome the localization mechanisms of the hypersensitive hosts? At present these questions cannot be answered.

It can be suggested, on the basis of all experimental results, that upon mechanical inoculation of a hypersensitive host, the limited supply of initial inoculum, followed by a rate of multiplication too low to penetrate physical or chemical barriers, or to exceed the rate of destruction of synthesizing virus, result in a delimitation and localization of the virus. If, however, conditions are established to make available an increased amout of inoculum, either through grafting, or by increasing the rate of multiplication through environmental changes such as temperature or nutrition, then the hypersensitive reaction can become systemic.—6660 N.W. Marine Drive, Vancouver, B.C., and Box 506, St. Catharine's, Ont.

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EFFECTS OF FUNGICIDES, OIL, AND FUNGICIDE-OIL-WATER EMULSIONS ON DEVELOPMENT OF CERCOSPORA LEAF SPOT OF BANANAS IN THE FIELD

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SUMMARY

Fungicides were protectant and antisporulant, whereas oil arrested further development of yellow streaks in Cercospora leaf spot of bananas in the field. In fungicide-oil-water emulsions, the fungicide component did not affect oil action and oil did not affect the fungicide except to improve coverage in the field. There were fewer yellow streaks in fungicide than in oil plots, whereas fungicide-oil-water emulsion plots had the fewest yellow streaks. In antisporulant studies, the majority of conidia formed on upper spot surfaces of untreated and oil sprayed leaves; in all fungicide treatments, the majority of conidia formed on the lower spot surfaces.

Bordeaux mixture and petroleum oils have been used extensively to control Cercospora leaf spot of bananas caused by *Mycosphaerella musicola* Leach (*Cercospora musae* Zimm.) (1, 2, 3). Bordeaux mixture has been effective in most areas, and sometimes unsatisfactory in others; petroleum oils have been effective, particularly with the streak-count system of spraying (3). Studies were initiated to analyze the specific effects of fungicides, oil, and fungicide-oil-water emulsions at various stages of the disease cycle in the field.

Review of literature.—Oil is not a protectant or antisporulant but arrests further development of yellow streaks and retards development of early brown streaks (3). Bordeaux mixture has a combined protective and antisporulant action. Calpouzos (1) observed that Bordeaux reduces conidial formation on the upper leaf surface but has little or no effect on the lower surface. He also found that Bordeaux mixture acts as a protectant, killing conidia falling on a sprayed leaf surface. Stahel (7) and Simmonds (6) observed that the protectant action is more effective if spray is applied to the lower rather than the upper surface of the youngest leaves. Leach (4) stated that Bordeaux mixture inhibits the germination of any conidia in dew rendered toxic by the chemical. According to Desrosiers (2), the effectiveness of fungicide-oil-water emulsions varies with the amount of oil, fungicide, and liquid applied.

The following observations by previous workers on disease development as related to control procedures were confirmed in preliminary studies. Stahel (7) and Simmonds (6) reported that the unfurled center leaf and the two youngest expanded leaves were most susceptible to *Cercospora musae*. The first symptoms, yellow streaks, appeared on the second-, third-, or fourth-youngest leaves. Furthermore, inoculating the lower rather than the upper leaf surface produced more spots. Leach (5) stated that nearly all invasion occurred on the lower surface of the center leaf before or just after unfurling. Desrosiers (2) determined that the majority of invasions were primary or from a dissemination period; during seasons of severe leaf spot, some secondary invasion occurred on the same leaves. Before then it was reported (3) that yellow streaks appeared in maximum quantities in specific areas at certain times rather than having a general daily occurrence. The time required for the various stages in the disease cycle was also reported (3).

Materials and methods.—All observations and materials collected were from Gros Michel plantations in Honduras in which cultural methods were standard unless otherwise specified.

The paraffin-base oils used had the following characteristics: Texaco 522—viscosity 99.3 SUS at 100°F, unsulfonated residue 85%, neutralization number 0.02, flash point (open cup) 370°F. Esso C—viscosity 74.8 SUS at 100°F, unsulfonated residue 93.4%, neutralization number 0.03, and flash point (open cup) 345°F. The oils were applied by Stearman biplane or Bell helicopter, at rates of 1.0–1.8 gal./A. Timing of applications was determined by the streak-count system (3).

Bordeaux mixture (10-7-100) with Triton X-114 (2 qt per 2000 gal.) and Marasperse C (2% of total solids) was applied from the ground at the rate of 200 gal./A. through a piping system and individually manned hoses on a 10–28-day cycle, depending on severity of *Cercospora* leaf spot and disease potential determined by past experience. The spray was directed to the center leaf, which was in various stages of unfurling.

Aerial-applied fungicides were C-O-C-S (basic copper chloride; basic copper sulfate), Dithane M-22 (manganous ethylenebis[dithiocarbamate]), Tri-basic (basic copper sulfate), and Microgel (basic copper sulfate with wetting agents). Application was by Stearman biplane or Bell helicopter at the rate of 10–12 gal. liquid per A., containing 5 lb of formulated chemical, 1.4 oz of Triton X-114, and water. Cycles were arbitrary and similar to those described for Bordeaux mixture. When Cercospora leaf spot was increasing, oil was included at rates of 0.5–1.0 gal./A. and the fungicide-oil-water emulsion was applied according to the streak-count system for that specific application. To formulate the fungicide-oil-water emulsion, varying amounts of oil replaced water, depending on the gal./A. of oil desired; 2% Triton X-45 or X-114 in the oil was used as the emulsifier.

To determine the effectiveness of protectant action using individual plants, all fungicides, with and without oil, were sprayed on portions of the upper or lower surfaces of unfurling center leaves. Bordeaux

mixture, prepared as described above, was sprayed with a DeVilbiss atomizer to simulated field conditions until runoff. The other fungicides and oil were sprayed not to runoff but to visible simulation of a 10–12-gal. and 1.5-gal./A. application by plane. Portions of other unfurling center leaves were controls. Symptoms, which appeared in 28 days, were from natural inoculum in the field. The number of yellow streaks on treated leaves was compared with the number on control leaves.

Results.—*Protectant action of fungicides and fungicide-oil-water emulsions.*—When Bordeaux mixture was hand-sprayed on upper or lower surfaces of the center leaves, control was highly variable, although generally less than from application to the lower surface. When sprayed on both surfaces, control was about 100%. Results were similar with C-O-C-S, Tri-basic, Microgel, and Dithane M-22.

Oil was evaluated by hand application as a protectant, both alone and in fungicide-oil-water emulsions. Oil alone did not reduce numbers or yellow streaks, whereas fungicide-oil-water emulsions gave the same protection as fungicides alone.

Observations on disease control were made under large-scale field applications. Visible coverage of Bordeaux mixture was present on most exposed surfaces of unfurling center leaves. Yellow streaks were consistently fewer in Bordeaux plots than in oil plots. With C-O-C-S, Microgel, and Tri-basic applied by airplane, visible coverage on the unfurling center leaf and youngest expanded leaf was not uniform and was sometimes sparse. Addition of oil in the form of fungicide-oil-water emulsions improved distribution on young leaves and increased the number of unfurling center and first youngest open leaves with some visible fungicide. Yellow streaks ranging from 100 to 2000 in number were observed on 50% of the individual leaves sampled in each of the aerial fungicide treatments at various times. In general, in experimental plots in which oil, fungicide, and fungicide-oil-water emulsion treatments were replicated, yellow streaks were fewer in the fungicide than in the oil plots, and fewest in the fungicide-oil-water emulsion plots.

Since added oil did not affect the protectant action of fungicides with optimal coverage, a study was initiated to determine if the fungicide component affected the action of oil in fungicide-oil-water emulsions applied to leaves with yellow streaks. Bordeaux and the other fungicides, with and without oil, were sprayed by hand on portions of leaves with yellow streaks. Portions of untreated leaves were used as controls. The numbers of arrested and developing streaks were recorded. Oil alone arrested 100% of streaks; fungicide alone did not affect yellow streaks, and they developed into spots; fungicide-oil-water emulsions gave disease control equivalent to that with oil alone, indicating that the fungicide component did not affect oil action.

Rates of oil were varied in fungicide-oil-water emulsions to determine optimal quantities for disease control. As the quantity of oil was increased in Bordeaux-oil-water emulsions from 0.5 to 1.0 gal./A., control of streaks became greater and more uniform. Streaks arrested were 0–71% at 0.5 gal./A., 41–100% at 0.75 gal./A., and 85–100% at 1.0 gal./A. Trends were similar with the other fungicides under these conditions. Under production practices using airplanes, however, a lower quantity and less uniform visible distribution of chemical is deposited on individual leaves. Applied by helicopter or biplane, oil alone at 1 gal./A. arrested 49–100% of the streaks (mean 92%); at 1.35 gal./A. arrested 73–100% (mean 95%); at 1.6 gal./A. arrested 85–100% (mean 97%); and 1.8 gal./A. arrested 98–100% (mean 99%). Random observations of Bordeaux-oil-water emulsions, not applied specifically at the time of maximum yellow streaks, indicated that 0–40% of streaks had been arrested and 15–65% of streaks had been retarded. The C-O-C-S, Microgel, and Tri-basic–oil–water emulsions were applied to yellow streaks at the optimum time for disease control. Oil rates were 0.5, 0.75, 1.0, and 1.25 gal./A., respectively arresting 33, 41, 53, and 60% of streaks (range 5–98%).

In a few cases, when fungicide-oil-water emulsions were applied to yellow streaks, rain fell before application was completed. Under these conditions, the control range was 0–27%, averaging 8%. By contrast, oil alone applied immediately preceding rain gave a variable percentage of arrests, varying with quantity of oil used regardless of rainfall.

Antisporulant action of fungicides.—The location and relative quantity of conidial formation on spots from untreated and treated leaves was determined. Leaves were brought into the laboratory, and spots in the optimal stage of development for conidial formation were cut out, placed on moist filter paper in Petri plates, and incubated 24–36 hours at 26°C. The spots were then examined under a dissecting microscope. In the untreated control and oil-sprayed leaves, the majority of conidia were found on upper surfaces; in all of the fungicide treatments, the majority of conidia formed on the lower surfaces (Table 1).

To determine the actual antisporulant effects of the various compounds in the field, leaves were treated as described above but spots were rated only as to presence or absence of conidia, regardless of surface. Untreated and oil-sprayed leaves were used as controls. In general, the fungicides applied under field conditions were all similar in effectiveness (Table 2). Antisporulation averaged about 20%. There was no apparent relationship of percentage of antisporulation and the interval of time between oil or fungicide application and leaf collection. Effect of fungicide-oil-water emulsions on sporulation was the same as that of fungicides alone.

During the conidial formation studies, some of the conidia appeared to have lifted up or penetrated the fungicide deposit as they developed. At early stages

of conidial development the fungicide was still in solution and not present as a crust. Germination of the conidia in contact with the fungicide was tested. After conidia had formed on the sprayed leaves, they were removed in a drop of water from the upper and lower spot surface and transferred to portions of untreated unfurling center leaves placed in Petri plates. The samples were incubated 4–7 days at 26°C. In these tests, the fungicide had been applied to the upper leaf surface in the field during farm-scale spray applications. Germination of conidia taken from the lower spot surface of fungicide-sprayed leaves did not differ from that of conidia from unsprayed leaves. Conidial germination from the upper surfaces of fungicide-sprayed leaves varied from 0 to 100% per spot, averaging 50% less than that of conidia from unsprayed leaves. Cell wall thickening was observed in individual cells of many of the conidia that did not germinate; chlamydospore-type cells also formed.

Discussion.—Texaco 522 and Esso C oils were effective in disease control after symptoms developed, and then only at the yellow streak stage; there was no apparent protectant or antisporulant effect. Thus the effectiveness of oil alone would depend on distribution of oil on the leaf and proper timing of application.

In contrast to the action of oil, Bordeaux mixture and other fungicides were effective only as protectants and antisporulants. Under current practices in Honduras, Bordeaux mixture applied from the ground can be sprayed only on limited areas at any specific time. If there were a general dissemination of inoculum over a large area, only limited areas could be protected. Since the unfurling center leaf of an individual plant may be in any stage of development when the fungicide is applied, the leaf surface area protected also varies among individual plants. Theoretically, until the efficiency of protection is improved, disease incidence will depend on inoculum potential, which will depend in part on antisporulant action. When the inoculum potential is low, the combined effects of protection, antisporulation, and lowered conidial germination are sufficient for effective control. When the inoculum potential is high over a long period, however, more leaf spots develop even though the percentage control is the same, because greater numbers of conidia are involved.

Aerial fungicides can be applied to large acreages at a specific time. Coverage of the youngest leaves, however, has been much poorer with aerial application of fungicides than with ground-sprayed Bordeaux mixture. As a result, more unprotected susceptible leaf surface remains exposed to the pathogen. Thus, the use of oil with present aerial fungicides will probably be necessary at times to maintain adequate control until the efficiency of protection and antisporulation of fungicides is improved.—Department of Plant Pathology, Vining C. Dunlap Laboratories, Tela Railroad Company, (subsidiary of United Fruit Company), La Lima, Honduras, Central America.

Table 1.—Effect of various treatments on the frequency and distribution of conidial formation on Cercospora leaf spots on upper or lower surfaces of banana leaves.

Treatment	Sporulation[a] of 200 spots per treatment			
	None	Mostly lower[b]	Mostly upper[c]	Abundant[d]
Untreated control	4	12	48	136
Esso C Oil	8	24	60	108
Bordeaux	12	148	8	32
COCS	12	136	4	48
Dithane M-22	16	116	24	44
Tri-basic	16	132	12	40
Microgel	16	120	16	48

[a] Sporulation rated "sparse" if fewer than 5 sporodochia had conidia and if there were fewer than 100 conidia per spot surface; sporulation rated "abundant" if reverse was true.

[b] None or sparse on upper surface; sparse or abundant on lower surface.

[c] Abundant or only on upper surface; none or sparse on lower surface.

[d] Abundant on both upper and lower surfaces.

Table 2.—Effectiveness of various fungicides as antisporulants in the field.

Days between application and collection	Fungicide	No. spots sporulating (50 reps)	% spots[a] not sporulating
3	Bordeaux	41	18
	C-O-C-S	40	20
	Microgel	34	32
	Tri-basic	43	14
	Dithane M-22	39	22
5	Bordeaux	31	38
	C-O-C-S	28	44
	Microgel	34	32
	Tri-basic	25	50
	Dithane M-22	33	34
9	Bordeaux	37	26
	C-O-C-S	37	26
	Microgel	44	12
	Tri-basic	47	6
	Dithane M-22	44	12
11	Bordeaux	47	6
	C-O-C-S	47	6
	Microgel	47	6
	Tri-basic	50	0
	Dithane M-22	46	8

[a] Corrected for control = 100% sporulations.

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GREASY SPOT AND TAR SPOT OF CITRUS IN FLORIDA

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SUMMARY

Greasy spot of citrus has at one time or another been attributed to rust mites *(Phyllocoptruta oleivora)*, physiological factors, fungi, and an unidentified organism. The present work confirms the report that a species of *Cercospora* is responsible. Tar spot affecting leaves and fruits is considered to be distinct from greasy spot and potentially a more serious disease. Names are proposed for the two species causing these diseases: respectively *C. citri-grisea* and *C. gigantea.* Oil emulsion, copper, or zineb applied from mid-June to early August gave satisfactory control of greasy spot. The combination of oil-zineb gives some nutritional effect and provides control of scale insects, greasy spot, and fruit russet.

Introduction.—Greasy spot, also known as greasy melanose and black melanose, may have first been recognized in Florida as early as 1896 (23), but not until 1915 (7) was a distinction made between it and true melanose (caused by *Diaporthe citri* Faw.). Since then, various workers have attributed greasy spot to rust mites (*Phyllocoptruta oleivora* (Ashm.)), physiological defects, fungi, and an unidentified organism. The disease has been reported from other areas, including the Azores, California, Central and South America, Ceylon, China, India, Italy, Japan, Java, Philippine Islands, Spain, Texas, Tunisia, West Indies, and Western Australia. Until the late '40s greasy spot was not regarded as a serious problem in Florida. Affected leaves could be found on almost every tree, but no marked defoliation occurred that could be attributed to the disease. During recent years, however, greasy spot has caused severe defoliation in many groves. In the late '40s, many growers began to use parathion instead of oil emulsion for the control of scale insects (*Chrysomphalus aonidum* (L.) and *Lepidiosaphes beckii* (Newm.)). An increase of greasy spot coincided with the decreasing use of oil emulsion sprays. In general, groves receiving summer oil emulsion had less greasy spot the following fall and winter than did groves sprayed with parathion. By 1952, many groves had lost foliage in amounts varying from a few leaves to about 95%.

This study was made primarily to determine the cause of greasy spot and to find effective and practical control measures. The causal agent and its chemical control are herein described.

Review of the literature.—Bitancourt (3) in 1940 wrote that rust mites always preferred to attack tissues where chlorophyll was in part destroyed as a result of some disease. In his opinion, greasy spot resulted from mites feeding on yellow spots of gum-infiltrated tissues often present on the lower surface of the leaves. In 1955 he said (in personal correspondence) that these gum spots were probably of physiogenic origin, possibly arising in connection with nutritional troubles. On the basis of field observations, Thompson (25) considered greasy spot to be the direct result of rust-mite feeding.

Fawcett and Klotz (9) reported that similar symptoms were seen in certain California locations where rust mites were not known to occur. Thompson et al (27, 28) later found greasy spot in Florida in the absence of rust mites; they further found no greasy spot where heavy infestations of rust mites were maintained on potted seedling grapefruit trees in a greenhouse.

Although no causal agent was isolated, early workers (20, 21) considered greasy spot to be the effect of an unidentified organism. In 1952, Tanaka and Yamada (24) reported inoculation tests showing that greasy spot was caused by *Mycosphaerella horrii* Hara (Syn: *Cercospora penzigii* Sacc. = *C. fumosa* Penz. = *C. aurantia* Heald & Wolf) and *Cercospora* sp.

Although greasy spot is known to be a fungus disease, the frequent rust mite-greasy spot association remains to be adequately explained.

Fig. 1.—Leaves naturally and artificially infected with greasy spot. Center leaf, natural infection.

There has been more agreement among workers on chemical control of greasy spot. The report cited by Fawcett (8) in 1936 that Japanese citrus growers were controlling the disease with Bordeaux was confirmed by Tanaka and Yamada (personal communication). Greasy spot has been controlled in Florida with copper-containing materials (10, 14, 26), zineb (zinc ethylenebis-[dithiocarbamate]), captan (*N*-(trichloromethylthio)-4-cyclohexene-1,2,-dicarboximide), ziram (zinc dimethyldithiocarbamate), nabam (disodium ethylenebis [dithiocarbamate]) plus zinc sulfate, and oil emulsion (10, 11, 12, 27, 28).

Symptoms of greasy spot.—Greasy spot chiefly affects the leaves of citrus, but on some varieties it attacks small twigs. It has a dark, slightly raised appearance, suggestive of a globule of dark grease under a semitransparent epidermis (Fig. 1). It occurs as more or less circular or somewhat irregular areas on both surfaces of the leaf, but more prominently on the lower. Affected areas often coalesce, giving the leaf a blotched appearance. Spots vary in color and pass through a transition from light yellow or mere translucence of the tissues through various shades of brown to black. Affected leaves are often conspicuously yellow when viewed from the upper surface. Occasionally, lesions develop into depressed, necrotic areas producing conidiophores and conidia (Fig. 3). This type of symptom has been called small, brown, round spot (24).

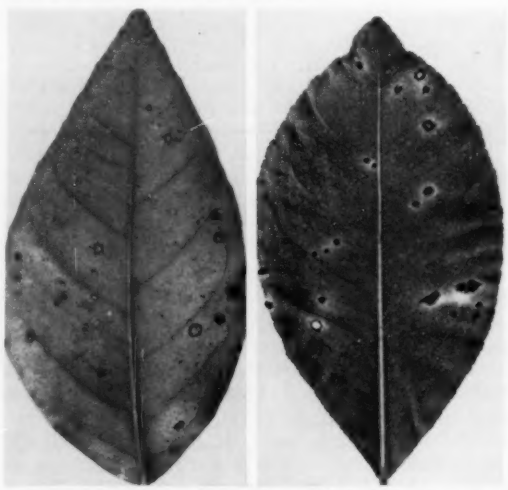

Fig. 3.—Small, brown, round spot. Left, Valencia orange leaf shows transition from small greasy spots. Right, Rangpur lime is an example of small, brown, round spot, in which case greasy spot symptoms were very short-lived.

Trees of all varieties and ages appear to be subject to infection. In general, however, grapefruit is more severely affected than orange, and young trees are more susceptible than older trees. Lemon may be damaged more than any other type of citrus. Although infected leaves can be found throughout the year, disease incidence is higher during late fall and winter than in spring or summer. In experimental plots in 1957, trees severely affected with greasy spot were more severely defoliated following freezing temperatures than trees with satisfactory greasy spot control.

Symptoms of tar spot.—Tar spot is the name here given to a disease (Fig. 2) that resembles greasy spot in being dark, raised, and "greasy" but affects fruit (Fig. 2) as well as leaves. With transmitted light, leaf symptoms of tar spot can easily be distinguished from greasy spot by a mahogany-red circle inside the periphery of the spot. The typical yellowing of greasy spot has not been observed on leaves affected by tar spot. In artificial inoculations (and under field conditions on leaves with iron deficiency), small, circular pits of tan to orange appear initially. Some of these spots grow rapidly; others develop as small scabby or warty growths that are whitish to light tan.

Tar spot is not now general in Florida, and is commercially of small importance. Inoculation tests have shown, however, that it is not only capable of causing leaf injury but can also kill seedlings. Because tar spot attacks fruits as well as leaves, it may be a more serious threat than greasy spot.

Greasy spot isolations and inoculations.—In 1946, a fungus, later proved to be pathogenic, was found in free-hand sections of greasy spot lesions stained with cotton blue. Leaves with affected spots were collected periodically, and isolations were attempted on potato-dextrose agar (PDA). Bits of leaf tissue with an entire disease spot were cut out, disinfected by dipping in mercuric chloride, rinsed with sterile distilled water, and placed on PDA. *Colletotrichium gloesporioides* Penz. was almost invariably isolated not only from diseased tissue but also from tissue of leaves that appeared healthy. This led to the assumption that *C. gloesporioides* was omnipresent on leaves and that a different technique would be necessary. Accordingly, attempts were made to isolate a pathogen by removing raised portions of spots by slicing longitudinally with

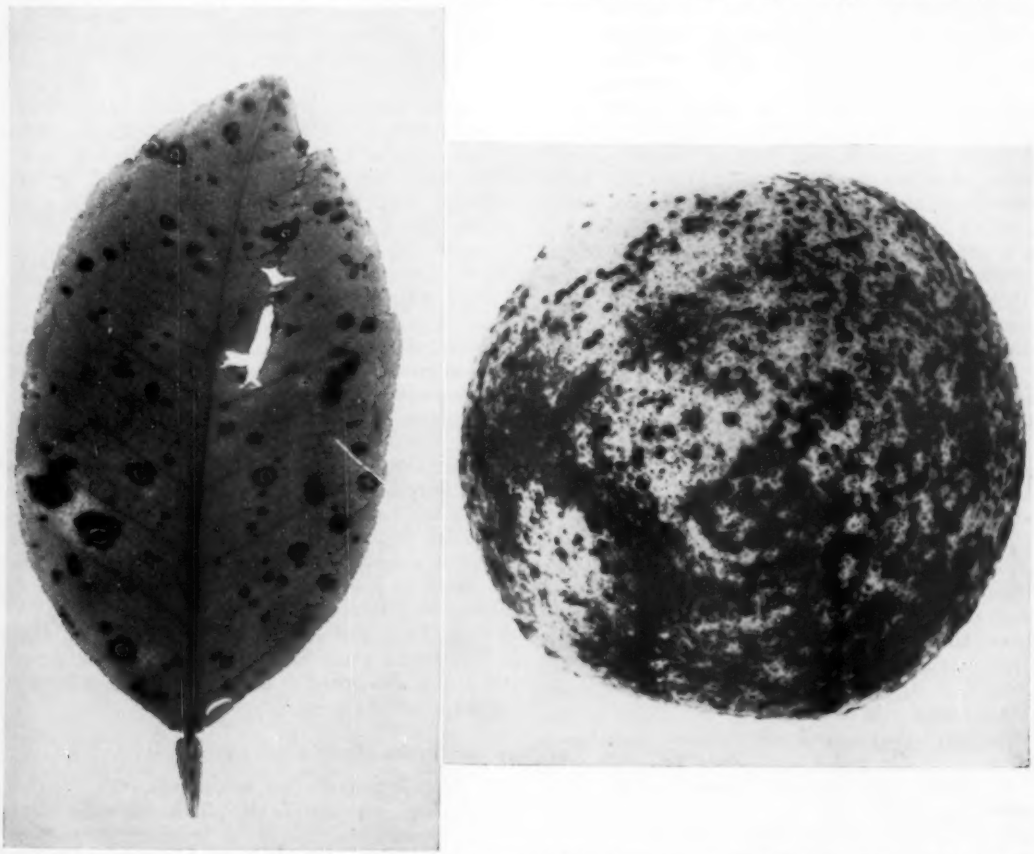

Fig. 2.—Tar spot. Left, leaf photographed with transmitted light shows several manifestations of disease. Note halo inside periphery of mature spots. Right, tar spot on Pineapple orange.

a razor-sharp scalpel and then removing minute sections of the underlying mesophyll tissue with very finely pointed watchmaker's tweezers. Resulting plates yielded a dark-gray to light-colored mycelial growth, which later proved to be *Cercospora*.

Grapefruit seedlings were grown aseptically in test tubes on nutrient culture media and, after attaining maximum size possible in tubes, were planted in 6-in. clay pots filled with soil previously treated with chloropicrin. The pots were placed in 2 sterilized, fluorescent-lighted, temperature-controlled (70°F) chambers. Since laboratory tests had shown that time for spore germination could be reduced from 24 to 12 hours by adding a small amount of citrus honey, all inoculations were made with a spore suspension in sterile water to which honey was added. Spore suspensions were sprayed on the lower leaf surfaces; plants were placed in a moisture chamber for 72 hours and then returned to the original chamber. A few water-soaked lesions appeared on some inoculated plants within 10 days to 2 weeks. Black areas developed in the lesions and the leaves began dropping by the end of the third week. Within 2 months each inoculated plant had lesions on one or more leaves.

Isolations of *Cercospora* from the inoculated leaves mentioned above were identical to the species used in the original inoculation. When spores from these isolations were inoculated into healthy plants, symptoms of greasy spot were produced. In 5 separate tests, 45 plants inoculated and held in temperature-controlled chambers developed typical greasy spot symptoms while 18 controls remained healthy. In 8 separate tests, 255 of 282 plants inoculated and held in a greenhouse became diseased while 111 control seedlings remained healthy. Time required for appearance of symptoms on plants held in the greenhouse varied from 6 weeks to more than a year after inoculation.

Leaves affected with greasy spot, both artificially and naturally infected, show identical patterns of development and color when photographed with transmitted light (Fig. 1). Lesions on the artificially infected chamber-held plants, however, did not become raised in the manner typical of lesions on naturally infected trees.

The reasons are not known for the difference in appearance between the rapidly developed, atypical lesions in the indoor chambers and the slowly developed, more nearly typical lesions in the greenhouse. From all tested spots, however—whether typical, atypical (insofar as not being raised), naturally infected, or artificially infected—*Cercospora* has consistently been isolated.

Tar spot isolations and inoculations.—Isolations of a species of *Cercospora* distinctly different from the one causing greasy spot were made from grapefruit and orange leaves and fruits displaying "tar spot" symptoms. Inoculations were made on grapefruit seedlings by the method used for greasy spot. Infection was obtained in 24 hours. Initial symptoms were evident when plants were removed from the moisture chamber. Plants maintained for a 72-hour period were so severely attacked that 20 of 23 lost all leaves and died. The organism recovered from the experimentally infected leaves, cultured and inoculated into healthy grapefruit seedlings, produced symptoms apparently identical to those in the original inoculation. This establishes the pathogenicity of the second species of *Cercospora*.

The causal agents of greasy spot and tar spot.—Neither species of *Cercospora* conforms to the description of *C. penzigii* Sacc. (*C. fumosa* Penz. and *C. aurantia* Heald & Wolf) as reported by Tanaka and Yamada (24) in Japan. To facilitate reference to these 2 distinct species, which do not appear to conform to species previously described, the names *Cercospora citri-grisea* and *C. gigantea* are proposed.

The name *Cercospora citri-grisea* is given to the species causing symptoms commonly referred to as greasy spot on its hosts in Florida.

Cercospora citri-grisea sp. nov.

Maculis rotundis vel subrotundis, tumidis, in foliis et interdum ramulis, similibus globulis olei fusci; laesionibus interdum depressis mortuisque et fructificationes gerentibus; stromatibus brunneis-subnigris; conidiophoris in fasciculis, olivaceis, non ramosis, amphigenis 4–6 × 60–80 μ conidiis tenuibus, plerumque indistincte multiseptatis, interdum non septatis, olivaceis, rectisrecurvis; basi longa obconice truncata; apice subacuto, 1.5–3 × 25–200 μ. Hab. in foliis, interdum in ramulis *Citri sinensis* (L.) Osbeck, *C. paradisi* Macfadyen, *C. limon* (L.) Burmann, *C. aurantifolia* Swingle, *C. reticulata* Blanco, et *Fortunella* sp. prope Babson Park, Florida, Amer. Bor., Jan. 15, 1958.

Forming more or less circular raised spots on leaves, and sometimes twigs, suggestive of a globule of dark grease. Lesions occasionally become depressed and necrotic, and produce fruit structures. Stroma dark brown to black; conidiophores fasciculate, olivaceous, unbranched, amphygenous 4–6 × 60–80 μ; conidia slender, occasionally one-celled, generally indistinctly multiseptate, olivaceous, straight to strongly curved, long obconically truncate base, subacute tip, 1.5–3 × 25–200 μ. On leaves and sometimes twigs of *Citrus sinensis* (L.) Osbeck, *C. paradisi* Macfadyen, *C. limon* (L.) Burmann, *C. aurantifolia* Swingle, *C. reticulata* Blanco, and *Fortunella* sp. Type locality: Babson Park, Florida, *(C. limon)* January 15, 1958.

The fungus commonly found to be associated with tar spot is designated as *Cercospora gigantea* because of the width of the spore.

Cercospora gigantea sp. nov.

Maculis rotundis vel subrotundis, depressis aut interdum paulo tumidis, in foliis ramulisque; maculis rotundis tumidisque in fructibus; laesionibus moribundis, 2–10 mm, fructificationes expedite gerentibus; stromatibus brunneis-nigris 60–90 μ;

conidiophoris brunneis, in fasciculis, 3–6 septatis, non geniculatis, non ramosis, amphigenis 4–6 × 90–160 μ; conidiis latis, 3–12 septatis, olivaceis, olivacesis-brunnels, rectis; basi truncata; apice rotundo, 6–8 × 80–180 μ. Hab. in foliis, ramulis, fructibus *C. sinensis* (L.) Osbeck et *C. paradisi* Macfadyen prope Winter Park, Florida, Amer. Bor., May 28, 1957.

Forming more or less circular, depressed or occasionally somewhat raised spots on leaves and twigs, and raised circular spots on fruit. Lesions becoming necrotic, 2–10 μ, readily forming fruiting structures. Stroma dark brown to black 60–90 μ; conidiophores, brown, fasciculate, 3–6 septate, not geniculate, unbranched, amphygenous 4–6 × 90–160 μ; conidia broad, 3–12 septate, olivaceous-brown, straight, base beveled, tip rounded, 6–8 × 80–180 μ. On leaves, twigs, and fruits of *Citrus sinensis* (L.) Osbeck and *C. paradisi* Macfadyen. Type locality: Winter Park, Florida, May 28, 1957.

Type specimens of *Cercospora citri-grisea* and *C. gigantea* have been deposited in the Herbarium of the University of Florida, Gainesville, Florida.

Chemical control.—In the summer of 1953, preliminary tests were begun to determine whether copper-containing sprays would control greasy spot in Florida. A block of Duncan grapefruit trees was divided into 4 plots of 7–12 trees each, including a nonsprayed control plot. One plot was sprayed on July 15, one on August 24, and one on May 2, June 23, August 3 (copper-oil emulsion), and November 17. Evaluations in the winter, by a method devised by Croxall *et al* (5) for the assessment of apple scab infections, are expressed in terms of percentage total leaf area infected. In the present work, however, 10 branches rather than 5 were used. Results of this test showed greasy spot infection to be reduced from 20% in the nonsprayed controls to 1% or less in the sprayed plots.

Because the use of copper on citrus in Florida leads sooner or later to toxic accumulations in the soil (19, 22), subsequent field tests were designed to find other materials that would control greasy spot. Table 1 shows results from a screening test in 1954 and timing tests in 1955 and 1956. Good commercial control was considered to be 0–2%. Greasy spot infection higher than 2% resulted in leaf drop; treatments that permitted more than 2% greasy spot were therefore considered unsatisfactory. Timing of application was important. In 1955, a treatment applied at the middle or the end of July was more effective than one applied June 29. Although zineb treatments in 1956 were effective throughout July and the first of August, control was greater from a treatment on the first or middle of July than from one applied the first of August. The reason may be that the rainy season began about 6 weeks earlier in 1956 than in 1955. Under excessive moisture conditions throughout the spring and summer of 1957, control measures applied between June 15 and the first part of July gave better control than did applications made between July 15 and August 1. However, an additional treatment in September would have been necessary for control in some groves.

To obtain the maximum protection from greasy spot, it would have been necessary to apply a treat-

Table 1.—Average percentage leaf surface area of grapefruit and orange affected by greasy spot following various treatments. Based on field examination of 10 branches per tree. Tests in 1955 and 1956 consisted of 4 tree plots randomized and replicated 3 or 4 times. All figures rounded to nearest whole number. Each experimental plot received petal-fall applications of copper-containing materials for melanose control.

Materials used[a] (lb and/or gal./100 gal.)	1954 Duncan grapefruit June 14 July 20 Aug. 17 Sept. 21	1955 Pineapple orange June 29	July 15	July 28	Valencia orange June 29	July 14	Aug. 2	1956 Ruby Red grapefruit July 2	July 16	Aug. 3	1956 Duncan grapefruit July 17	1956 Pineapple orange July 6
Zineb, 0.65								0	0	1	0	0
Zineb, 1.30	0	6	2	0	1	0	0	0	0	1	0	0
Oil, 1.3%	0[b]	4	1	0	1	0	0					
Oil, 0.7%, & parathion, 0.15								2	1	4	5	13
Oil, 0.7%, & parathion, 0.15 & zineb, 0.65								0	0	1	0	0
Basic copper, 0.84	0	11	6	2	1	0	0					
Ziram, 1.14	1[c]	11	8	5	7	4	2					
Captan, 1.0	0	24	9	8	7	5	9					
Parathion, 0.15, & w. sulfur, 5		10	9	9	6	8	7					
Lime-sulfur, 1, & w. sulfur, 5		19	19	15	12	10	8					
Neomycin, 75.7 g	5											
Nabam (1 qt), 0.19 & zinc sulfate, 0.75	0											
Non-sprayed	25	14			11			13			14	25

[a] Zineb was used as a 65% formulation; oil as a 90% formulation; parathion as a 15% formulation; copper as a 56% formulation; ziram as a 76% formulation; captan as a 50% formulation; and zinc sulfate as a 36% material.

[b] 1 application on July 20.

[c] 3 applications on June 1, July 1, July 29, on Pineapple orange.

ment after the expansion of each flush of growth. Time of growth flushes, of course, varies from year to year depending upon environmental conditions.

Observations in a Valencia orange grove in the spring of 1957 indicated that zinc contained in zineb sprays is available for plant nutrition. Spring growth exhibited zinc deficiency symptoms on non-sprayed trees but not on trees that had received zineb the previous summer. Following a spring application of zineb (0.65 lb/100 gal.), the zinc-deficient leaves developed a normal color. In another grove, 0.65 lb zineb as a spring application was as effective in reducing deficiency symptoms as was 1 lb zinc in sulfate form. These results are of particular importance to the grower in that zineb can be added to the regular summer oil and parathion treatment, eliminating summer applications of sulfur that would normally be applied to control russet (13).

Fungicidal control of tar spot was not attempted. Observations indicate separate leaf and fruit infective periods and suggest that more than one spray would be required for control.

Discussion.—Several factors may be involved in variations of susceptibility. There is the possibility of physiological differences in growth patterns or rate between young and old trees.

Young trees widely separated and exposed are generally more seriously affected than older, crowded trees. On older, uncrowded trees the south or southeast sides frequently are more severely attacked than other portions. In most groves, regardless of age or spacing, branches defoliate more readily in tree tops than below. Of course, the fact remains that it is difficult at best to obtain adequate spray coverage in the tops of tall trees.

With citrus Bahgat (1) found the density of stomata on the leaves of a given species to be greatest in hot arid regions and least in cool moist regions and that shading of the leaves reduced the density of stomata and increased their size. If incidence of greasy spot is correlated with the number of stomata, the greater infection on leaves receiving maximum sunlight, and hence high temperatures, would be explained, assuming other climatic factors such as moisture to be equal. Individual leaves partially turned or twisted have been observed to have more spots on the portion more exposed to light and moisture.

Physiological factors in the host also appear to play a role in the development of *Cercospora* spp. on citrus. In the field, it has often been observed that leaves exhibiting deficiency of minerals may be attacked more severely by greasy spot than leaves with a normal appearance. In one grapefruit grove, flushes of growth with iron-deficiency symptoms had more tar spot lesions than did flushes with leaves of normal appearance.

Other diseases such as systemic viruses may also be factors in the infection and development of *Cercospora* spp.

Low disease incidence on oil-sprayed trees has been attributed to both the physiological and fungistatic effect of oil. According to Merrin (18), oils affect the behavior of stomata in citrus so that the transpiration rate is affected for more than a month after treatment. From his results it would seem that any spray that reduces the transpiration rate would also interfere seriously with the metabolism of the plant. He assumed that the retarding effect of the oil sprays was a stomatal phenomenon since it is greater on older leaves, in which the stomata are probably fully developed and functional to the maximum degree. It appears to the writer that the actual metabolic rate of the plant may be also affected so that, in the case of fungus infection, the fungus not only may have difficulty in penetrating the stomata but also may find the physiology of the host so altered as to provide an environment unsuitable for its optimum growth.

Recent work in Guadeloupe (15) and Jamaica (16) has demonstrated that oil is also effective against *C. musae* attacking banana. Leach (17) reported on the fungistatic effect of oil after infection and during development of the hyphae in the mesophyll of the leaf.—University of Florida, Citrus Experiment Station, Lake Alfred.

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OAT VARIETIES WITH ADULT-PLANT FIELD RESISTANCE TO RACE 264 OF THE CROWN RUST FUNGUS

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SUMMARY

Large oat nurseries were grown in Puerto Rico during the winters of 1957 and 1958 to screen for resistance to the crown rust fungus *Puccinia coronata* var. *avenae*, race 264. Most entries grew well and were subjected to a heavy infection of race 264. Several hexaploid lines exhibited a high degree of adult plant-resistance in both tests, and others were moderately resistant. Certain progeny from crosses between resistant lines and susceptible lines with desirable agronomic qualities retained the resistance. A number of lines of noncultivated species of *Avena* were highly resistant.

The appearance and spread in North America of race 264 of crown rust (*Puccinia coronata* Cda. var. *avenae* Fraser & Led.), capable of parasitizing Landhafer and all other sources of seedling resistance known among oats (4), prompted a search for resistant varieties. A moderate degree of adult-plant field resistance has been reported in certain oat varieties (1). More recently (2) a number of lines showing a high degree of field resistance have been found.

The technique of using seedling reaction to known races of rust in greenhouse studies is not satisfactory for screening adult-plant field resistance to race 264. The reason is that plants known to have adult field resistance to race 264 may show moderate or complete susceptibility in the seedling stage. Tests for adult-plant reaction are difficult for a number of reasons: if made in the greenhouse, they are expensive and may not indicate true field behavior; if made in the field, they may endanger commercial oat plantings in the area and may be difficult to interpret because of contamination from naturally occurring races.

This report contains results of 2 field tests for adult-plant reaction of oats to race 264 conducted in Puerto Rico during the winters of 1957 and 1958. Puerto Rico was chosen for the test because: 1) oats are known to grow normally during the winter season, and severe infections of rust in test plots are easily established; 2) oats are not grown on the island commercially, and there is no problem of contamination with other races of crown rust; and 3) the distance from the mainland and the direction of prevailing winds make it unlikely that spores would be blown to North America or the reverse.

Table 1.—Average reaction of certain lines of hexaploid oats to *Puccinia coronata avenae* race 264 at Isabela, Puerto Rico, in 1957 and 1958.

C.I. no.	Name	Original source	Apparently similar or identical lines or synonyms		Reactions[a]	
			P.I. nos.	C.I. nos.	1957	1958
2848	Unnamed	Russia	None known	None known	MR	R
3032	S.E.S. Sel. 49	Africa	None known	None known	R	MR
4508	La Industria	Colombia	148980	None known	R	R
5083	La Estanzuela	Argentina	197279	4363, 5084, 7049, 7385	MR	MR
5105	Johnson	Virginia	None known	None known	MR	R
7147	S.E.S. Sel. 52	Africa	174545	3033, 3034	R	R
7171	D.L. 41372	Argentina	185783, 186269	7381	R	R
7172	D.L.M. 3	Argentina	197278	7055	R	R
7181	K.H.C.	France	174544	6665	R	R

[a] R, resistant; MR, moderately resistant.

Materials and methods.—The first nursery, consisting of 4804 entries, was planted in November, 1957, at Isabela, in the northwest corner of the island. These selections included 3750 entries from the World Collection of oats maintained by the U. S. Department of Agriculture. This collection includes strains of cultivated oats from 52 foreign countries, and about 150 strains of noncultivated species of *Avena* from widely distributed countries. The remainder of the entries in the nursery were lines of hybrid origin supplied by numerous oat breeders and pathologists in the United States and Canada.

The second nursery, consisting of 4700 entries, was planted at Isabela during the early part of December, 1958. A large portion of the material in this nursery was composed of breeding selections. The nursery also contained lines found or suspected to be resistant in the tests of the preceding year, some of which were used as regularly recurring checks throughout the nursery.

Spreader rows of the highly susceptible Markton oats surrounded the nursery and were inoculated with a hypodermic syringe at 6 weeks of age. The inoculum used was race 264, increased at Ames, Iowa, from a single pustule, with special precautions being taken to maintain purity.

Results.—Emergence of the seedlings, in general, was good. Some entries were destroyed in 1957 by a serious infestation of mole crickets (*Scapteriscus vicinus* Scudder) before control measures were effective. Subsequent growth of resistant lines with spring growth habit was excellent, comparing favorably with growth in the midwestern United States. Lines with winter growth habit did not head, and some showed a degree of premature senescence or death that interfered with rust observations.

Certain lines carrying the Victoria gene for crown rust resistance died in relatively early stages of growth, and spores similar to those of *Helminthosporium victoriae* Meehan & Murphy were observed. The cause of death appeared to be Victoria blight.

In both seasons crown rust developed in the spreader rows 10 days after inoculation and spread rapidly and uniformly throughout the nurseries. The infection was so severe that most susceptible lines were killed before progressing beyond the boot stage. In 1957, when a large number of susceptible entries of the World Collection were tested, it was estimated that 95% of the test lines were killed by crown rust before heading.

A few hexaploid lines that were resistant or moderately so were observed in the 1957 test (Table 1). Even though infection of susceptible plants in adjacent rows was heavy, the resistant lines usually showed only a small amount of flecking due to rust, and this flecking was difficult to distinguish from that due to other factors. Lines designated moderately resistant showed flecking and small pustules.

Three selections, C.I. nos. 7147, 7171, 7181, which proved to be resistant in the 1957 trials, were used as standard checks and planted at intervals throughout the 1958 nursery. These varieties appear to be the most useful of the resistant varieties, since they are known (2) to have field resistance to races of crown rust common in the United States. Once again, they were highly resistant throughout the nursery.

There were few changes of reactions of resistant selections from test to test. C.I. nos. 4508, 7172, and 7181 were highly resistant in both tests. C.I. 3032 was recorded as resistant in 1957 but only moderately so in 1958. Plants of C.I. nos. 2848 and 5105 all had small pustules in the first test but were resistant in the second test. C.I. 5083 was moderately resistant in both seasons.

Some of the resistant lines selected in 1957 were used in the development of material furnished by plant breeders and pathologists for the 1958 tests. Certain lines from these crosses showed resistance, some being as good as the original resistant parent. C.I. 7181, in particular, appeared in the parentage of lines with excellent resistance and reasonably satisfactory agronomic character.

A number of the lines in the "miscellaneous species" collection were resistant (Table 2) in both tests. These resistant selections include all of those reported (3) to be resistant in the seedling stage and others that were susceptible. The latter include the diploid entries C.I. nos. 3785 and 4749. Another diploid, C.I.

Table 2.—Average reactions of certain lines of noncultivated species of *Avena* to *Puccinia coronata avenae* race 264 at Isabela, Puerto Rico, in 1957 and 1958.

C.I. no.	P.I. no.	Species	Name	Source	Chromosome no. (2n)	Reactions [a] 1957	1958
2630	78821	*A. strigosa*	Glabrota	Australia	14	R	R
2525		*A. strigosa*		Wales	14	R	R
3785		*A. strigosa*		Poland	14	MR	R
3815		*A. strigosa*		Poland	14	R	R
4639		*A. strigosa*	Saia	Brazil	14	R	R
4746	158246	*A. strigosa*		Spain	14	R	R
4747	158245	*A. strigosa*		Spain	14	R	R
4748	158247	*A. strigosa*		Portugal	14	MR	R
4749	158204	*A. brevis*		Portugal	14	MR	R
7232		*A. abyssinica*	Abd. Sp 101	U. S.	28	R	R
7233	193958	*A. abyssinica*		Ethiopia	28	MR	MR

[a] R, resistant; MR, moderately resistant.

4748, which was intermediate in the greenhouse, had resistance in the field.—Federal Experiment Station, Territorial Experiment Stations Division and Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture.

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A LEAF SPOT OF COTTON CAUSED BY CORYNESPORA CASSIICOLA

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SUMMARY

A species of *Corynespora* pathogenic on 2 cotton species, *Gossypium hirsutum* and *G. barbadense*, in the Mississippi-Yazoo Delta area appears identical with one causing target spot of soybean and blight of sesame and agrees with the description of *C. cassiicola*. Isolates from cotton, soybean, sesame, and cowpea were cross inoculated in all combinations. The fungus overwintered on cotton stems in the field without loss of pathogenicity. A comparison with a similar cotton leaf-spotting fungus, *Helminthosporium gossypii*, showed the two pathogens to be distinct. Indications are that *Corynespora* leaf spot of cotton has been present in some areas for at least 25 years.

In August, 1959, a leaf spot of cotton, *Gossypium hirsutum* L., was observed in 3 commercial fields in the Mississippi-Yazoo River Delta. The locations were at Holly Bluff, Pace, and Stoneville, Mississippi; and the respective cotton varieties were Delfos 9169, Delta and Pine Land 15, and Delfos 8274. In addition, the leaf spotting was observed on several lines of *G. barbadense* L. in the experimental nursery at Stoneville.

The leaf spots were roughly circular, with light-brown centers surrounded by narrow dark-brown margins. Diameters of the individual lesions varied from 2 to 6 mm, but in many cases the lesions coalesced and produced larger irregular necrotic areas (Fig. 1-A).

A fungus was repeatedly isolated from the leaf spots. It fitted the description of *Corynespora cassiicola* (Berk. & Curt.) Wei (2, 6), the incitant of target spot of soybean, *Glycine max* (L.) Merr., and blight of sesame, *Sesamum indicum* L., in the Mississippi Delta area (4). This fungus has been observed on a large number of plant species (2, 6), but as far as the writer is aware has never been reported on cotton. Of all the cotton leaf spot fungi noted, only that described by Tucker (5) as *Helminthosporium gossypii* Tucker on *G. barbadense* resembled the organism in question. Since *C. cassiicola* had been pre-

viously described as a *Helminthosporium* (3), the two fungi were compared and found distinct. Details of the separation are presented below (experimental results).

It was further noted that the infected cotton was growing adjacent to fields with *Corynespora*-infected soybeans at Holly Bluff and Stoneville, and infected sesame at Pace. Because of the proximity of the infected crops and the similarity of the cotton fungus to *C. cassiicola*, the study reported was carried out to establish the identity and pathogenicity of the organism.

Materials and methods.—Four series of cross-inoculations were made. In the first three, isolates of the leaf-spotting fungus from cotton and *C. cassiicola* from soybean were compared on these 2 hosts; the fourth included isolates of *C. cassiicola* from cowpea, *Vigna sinensis* (Torner) Savi, and sesame with all 4 fungi and hosts in combination.

All the fungus isolates used except those from cotton were obtained originally at Stoneville, in August, 1959, by single-spore transfers from naturally infected hosts. The cotton isolates were similarly obtained at Holly Bluff. Isolates were maintained on tubed PDA (potato-dextrose agar).

The plant varieties used were Woods Yellow soybean; Margo sesame; Whiteacre cowpea; Hartsville, Delfos 8274, Delta Experiment Station line 7343, and Rex (*G. hirsutum*) and Seabrook (*G. barbadense*) cotton. Three or 4 plants per pot, were grown in potting soil in 6- or 8-in. pots. In each inoculation series 8–16 plants of each species were inoculated with the respective isolates, and 4–6 plants were uninoculated controls. Plants were 30 days old when inoculated except for the cotton plants of the third series, which were 107.

Inoculum was prepared from 10- to 14-day-old cultures growing on PDA in 9-cm Petri dishes. The cultures were macerated in distilled water in a food blender in a concentration of 1 culture per 200 ml of water plus 1 drop of wetting agent (Triton X-100). Plants were sprayed with inoculum until uniformly wet. Control plants were sprayed with a solution of sterile medium and wetting agent in the same concentration as the inoculum. The sprayed plants were placed in a mist chamber for 48 hours, then removed to a greenhouse bench and observed for symptoms.

The cotton leaf spot fungus was compared with Tucker's original description of *Helminthosporium gossypii* (5) and with his herbarium material. The herbarium material was lent through the courtesy of John A. Stevenson, formerly curator of the National Fungus Collections of the U. S. Department of Agriculture. It consisted of infected leaves and bracts of *G. barbadense* collected at Lajas, Puerto Rico, on February 15, 1924.

Experimental results.—*Cross-inoculation.*—The first cross-inoculation series included soybean and cotton (Hartsville and Seabrook) and their respective fungus isolates. Lesions were apparent on all inoculated plants within 2–3 days of inoculation, whereas control plants were disease-free. No difference was apparent in pathogenicity of the 2 fungi. Both were reisolated from both hosts.

The initial symptom on cotton was brick-red pinpoint lesions on the leaves. These lesions increased in diameter (2–4 mm) and developed tan centers and dark reddish-brown margins. On heavily spotted leaves, the lesions usually coalesced and formed large, irregular necrotic areas. This symptom was usually followed by leaf chlorosis and abscission. The lesions also occurred on the petioles and frequently resulted in girdling of those parts.

Symptom expression on soybean was similar to that on cotton. In addition to leaf-spotting, reddish-brown necrotic streaks occurred on stems, leaf petioles, and veins. The vein necrosis was principally confined to the underside of the leaf (Fig. 1-B). In the field, vein necrosis occurs on soybean leaves naturally infected with *C. cassiicola* and is a good diagnostic character for the disease.

The second inoculation series included soybean and cotton (Delfos 8274, D.E.S. 7343, and Seabrook). Inoculum was made up from the respective fungi reisolated from the inoculated cotton of the first series. The results of the inoculation were similar to those of the first series (Fig. 1-C). Reisolation was again made.

The third cross-inoculation involved soybean and 107-day-old cotton (D.E.S. 7343 and Seabrook). Results were likewise similar to those in the first 2 cross inoculation series. In addition, bract infection, expressed as reddish-brown, pinpoint lesions, was observed. It was apparent that the pathogenicity of the 2 fungus isolates was not limited to juvenile cotton but included mature plants as well.

The fourth cross-inoculation series included soybean, sesame, cowpea, and cotton (Rex), and their fungus isolates in all combinations. Infection was observed on all inoculated plants 2–3 days after inoculation. No difference in pathogenicity was noted among the 4 isolates. Reisolation was made from all 4 host plants.

All 4 fungus isolates produced the symptom response previously described in cotton and soybean. On sesame, dark-brown angular leaf spots increased rapidly in size, coalesced, and blighted the leaves. The plants were quickly defoliated in this manner and killed. Cowpea leaves showed only small brick-red lesions, 1–2 mm in diameter, which increased little in size. Heavily spotted leaves, however, turned gradually lemon-yellow and abscissed.

Overwintering.—Cotton stem tips (12–18 in.) were collected at random from overwintered stalks in 2 separate fields at Stoneville on March 28, 1960, placed in moist chambers for 48 hours, and examined with a stereoscopic microscope. Production of *Corynespora* conidia was abundant on 7 of 37 and 15 of 41 stems respectively. At this time 13 single-spore transfers to PDA produced 13 typical *Corynespora* colonies. To check the pathogenicity of the overwintered fungus

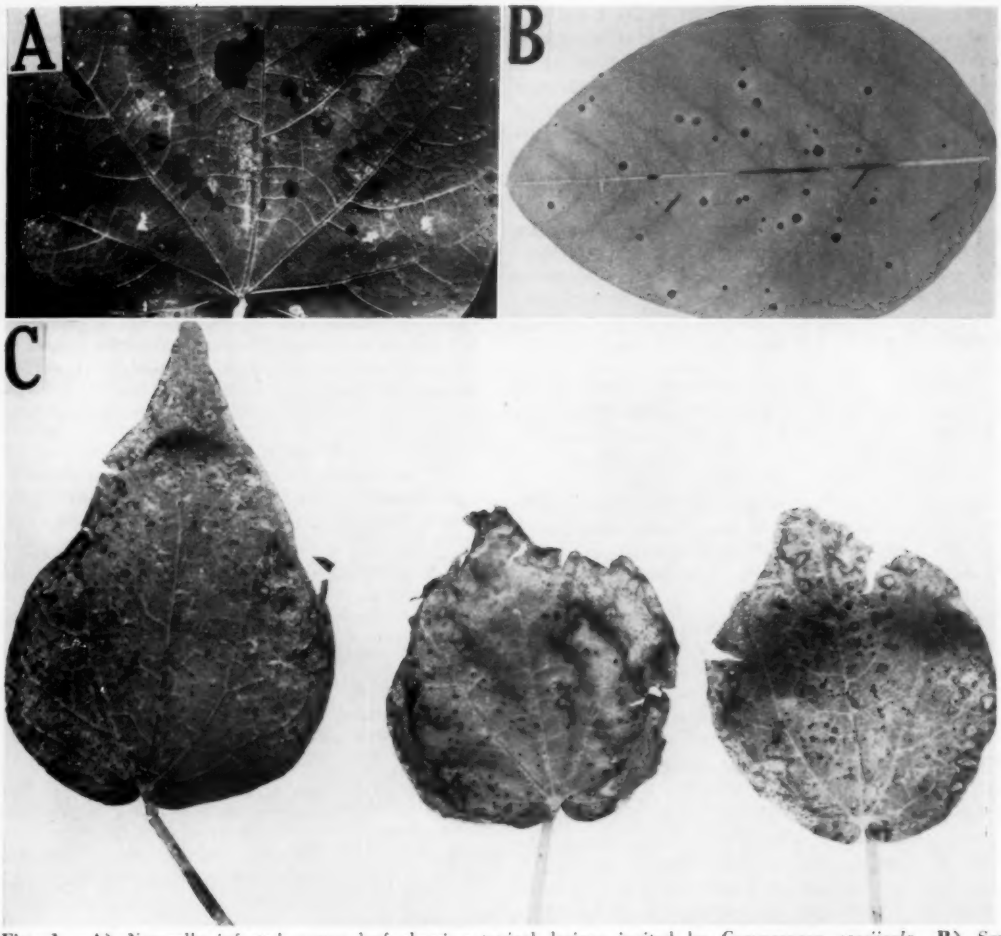

Fig. 1.—A) Naturally infected cotton leaf showing typical lesions incited by *Corynespora cassiicola*. **B)** Soybean leaflet illustrating leaf spotting and vein necrosis produced by inoculation with *C. cassiicola* isolated from cotton. **C)** Primary leaves of 3 cotton varieties with symptoms produced by inoculation with *Corynespora cassiicola* isolated from cotton. Left to right: Seabrook, D.E.S. 7343, and Delfos 8274.

further. 4 colonies were prepared as inoculum and sprayed on 4 plants each of soybean and cotton (Rex). Infection was excellent on both hosts. Evidently the fungus can overwinter in the Mississippi Delta area on cotton stems without loss of pathogenicity.

Comparison of Corynespora cassiicola from cotton with Helminthosporium gossypii.—Conidia and conidiophores of the leaf spot fungus on naturally infected cotton leaves were examined and measured. The dimensions of 50 conidia were 76.0–288.0×6.7–20.2 μ, averaging 135.1×14.1 μ. A like number of conidiophores measured 45.6–188.2×3.4–10.1 μ, averaging 111.1×6.6 μ. The conidia were mostly obclavate to pyriform, tapering gradually toward the apex. When these same leaves were placed in moist chambers for 24 hours, the conidia and conidiophores markedly elongated and the conidia became cylindrical. Catenulation of conidia also occurred. Both elongation and catenulation of *C. cassiicola* conidia under moist conditions were described by Wei (6) and Olive et al (3). Dimensions of the conidia produced in the moist chambers were 85.2–312.4×6.5–10.1 μ, averaging 187.6×9.0 μ, and of the conidiophores 129.2–516.8×3.4–6.7 μ, averaging 304.2–5.7 μ.

The conidial dimensions of *H. gossypii*, in contrast, were reported by Tucker (5) to be "quite constant whether produced under moist or dry conditions." Tucker's measurements were 35–118×11.7–18.4 μ, averaging 87×15.3 μ. Further examination of Tucker's illustrations and herbarium material revealed that the conidia of *H. gossypii* differed from those of *C. cassiicola* in morphology and mode of germination. Conidia of *H. gossypii* are elliptical, being widest in the middle portion, whereas those of *C. cassiicola* are obclavate-pyriform, being widest near the base. The conidial hilum of *H. gossypii* "is visible only under

high magnification, and is included in the basal contour," whereas that of *C. cassiicola* is prominent (3.6–7.9 μ diameter), annular, and not included in the basal contour. Both fungi exhibit bipolar germination of the conidia with production of germ tubes. Germination from the basal cell in *H. gossypii*, however, differs from that in *C. cassiicola* in that "a germ tube does not push through the hilum but through the base of the spore adjacent to it"; in the latter species the germ tube passes through the hilum (3, 6). One hundred germinating conidia of *C. cassiicola* from cotton were examined, and all were found to germinate through the hilum.

It is evident from the foregoing that *C. cassiicola* and *H. gossypii* are not identical.

Discussion. The evidence presented shows that the leaf spot fungus isolated from cotton is *Corynespora cassiicola* and is pathogenic to both *Gossypium hirsutum* and *G. barbadense*. Further, the cotton pathogen is identical with the one that causes target spot of soybean and blight of sesame in the Mississippi Delta area. It is interesting that the isolates from the 4 different hosts were alike in pathogenicity in view of the description of 2 separate races from cowpea and soybean (3). It was reported earlier (4) that isolates from soybean and sesame obtained in the Mississippi Delta did not fit the description of either race, thereby indicating an additional race. Since the 4 isolates described herein behaved alike and in the same manner as the earlier soybean-sesame isolates, it appears that only one race of *C. cassiicola* prevails in the delta area. Nevertheless the separation of races (3) was based on somewhat limited evidence, and distinct races as such may not exist. Further investigation is needed.

Comparison of the cotton leaf spot fungus with *H. gossypii* leaves no doubt that the 2 are distinct entities. Because of the similarity of the 2 fungi, however, previous reports of *H. gossypii* were re-examined to ascertain whether the fungus described may have been *C. cassiicola* in some instances. One such instance was found in a report by Clara, in 1935, of a cotton leaf spot fungus in the Philippines (1). Clara's excellent illustrations clearly indicate that the fungus was a species of *Corynespora*, very probably *C. cassiicola*. The conidium was obclavate, possessed a distinct annular hilum, and germinated from the basal cell through the hilum. Clara's report is evidence that *Corynespora* leaf spot of cotton is not a new disease but has been present in some areas for at least 25 years.—Delta Branch of the Mississippi Agricultural Experiment Station, Stoneville.

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EXISTENCE OF FUSARIUM SOLANI F. PHASEOLI AS CHLAMYDOSPORES IN SOIL

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SUMMARY

In naturally infested field soil the bean root-rot Fusarium (*Fusarium solani* f. *phaseoli*) exists chiefly in the form of chlamydospores as demonstrated by direct observation and by culturing methods. Smears on slides of untreated field soil readily showed Fusarium chlamydospores. When soil from bean fields in fallow, in beans, or in a rotation with another crop was cultured in plates of modified Martin's medium, Fusarium colonies appeared in 30–48 hours. Hyphal tips were transferred for identification while the rest of the colony was transferred and microscopically examined on a slide. The bean Fusarium colonies generally arose from a chlamydospore associated or imbedded in a fragment of a tissue or humus particle.

When macroconidia were seeded in unsterilized field soil they either germinated into a short germ-tube which eventually produced chlamydospores or were converted directly into chlamydospores. When sterilized soil was similarly seeded, the macroconidia germinated and the mycelium grew extensively through the soil and was eventually transformed into chlamydospores in a period varying from days to weeks. The chlamydospores observed in soils occurred singly, in pairs, or rarely, in chains, and varied in shape (round, subglobular, or pear-shaped), size (6–16 μ), and wall thickness.

None of 3700 isolates from field soil or of 350

isolates from individual lesions from field-grown infected plants yielded the mycelial (M) mutants common in single-spore cultures in the laboratory. Microscopic examination of free-hand and microtome sections of diseased tissues from naturally infected and from inoculated bean plants at various stages of growth revealed that chlamydospores were formed abundantly either on the surface or within the outer cortex of the necrotic lesions as the plant dies. The chlamydospores were of 2 types. Large ones, mostly on the surface of the host, were subglobular, single, in pairs or in chains of 3, with thick, warty walls and rich in oil droplets. Small ones, mostly in the intercellular spaces of outer cortex, were oval or pear-shaped, with smooth and thinner walls. Abundant macroconidia were occasionally produced on the diseased tissue at the soil-level under favorable conditions of moisture and light. These were readily converted into chlamydospores upon reaching the soil.

Little is known of the actual mode of existence of root-invading fungus pathogens in their natural soil habitats. This is no less true of the pathogenic soil-borne species of Fusarium in spite of extensive studies made of them on their various hosts throughout the world (frequent speculations notwithstanding).

In a previous paper (4) it was shown that *Fusarium solani* (Mart.) Appel & Wr. f. *phaseoli* (Burk.) Snyd. & Hans. is present in field soil in qualitatively distinctive clones, and that the root and foot rot of bean, *Phaseolus vulgaris* L., caused by this fungus commonly results from multiple infections of the underground parts of an individual plant by these different clones. These findings raise such questions as, in what form does *F. solani* f. *phaseoli* exist in the soil, i.e., as mycelium, conidia, or chlamydospores; does the fungus grow through the soil in search of a host; and do the mutant mycelial (M) types that arise so abundantly from sporodochial (C) types in the laboratory play a consequential role in the field? This paper reports investigations of these problems.

All soil used, untreated field soil unless otherwise specified, was stored indoors or outdoors in containers of various sizes. When sterilized, soil was either autoclaved or gassed with propylene oxide. *F. solani* f. *phaseoli* was added to soil in the form of culture-medium-free macroconidial suspensions in water.

Chlamydospores visible in soil suspension smeared on slides.—Observations on the behavior of the fungus in the soil were made by the soil smear technique. A small amount of the soil sample is mixed with water to make a slurry, which is poured on a glass microscope slide (1 or 2 drops at a time) in an amount sufficient to be covered by a large cover slip. Where germination of spores is not sought, acid fuchsin stain is added to the slurry in amounts required to give a deep red stain to the spores. The smears are examined under the high power of the compound microscope.

The use of this technique with sterilized or unsterilized inoculated soil revealed the existence of the fungus as chlamydospores. These chlamydospores generally occur singly or doubly, rarely in chains of 3 or more. They are round, subglobular, or occasionally pear-shaped, and vary in diameter from 6 to 16 μ. The larger ones usually have thick, rough, undulating walls, and contents that take the acid fuchsin stain deeply. Also, they contain numerous oil vacuoles, whereas the small chlamydospores, in contrast, have thin walls and possess rather uniform lightly staining contents with little or no oil evident in them.

Pure-culture identification of chlamydospores on water-agar-soil smear slides.—To make a semipermanent mount or to make a mount in which chlamydospores will germinate and can be isolated and cultured directly from the mount, a warm water-agar-soil suspension is made (3 g soil per 50 ml 1.5% water-agar), pipetted onto the glass slide 0.6 ml per slide), and spread quickly over the entire surface of the slide. No dye is added to the slurry. With this technique the chlamydospores, as well as the conidia, of *F. solani* f. *phaseoli* are readily observed, and their germination may be followed by placing the slides in Petri-dish moist chambers. From germinated spores on these slides, hyphal tips are cut off and transferred to plates of acid potato-dextrose agar (PDA), or to other appropriate media. By this procedure the structures seen in the soil smear may be correlated with the specific fungus involved.

On the other hand if the agar is allowed to dry down on the surface of the slide, it can be stained (aniline blue–acetic acid–phenol for 1 hour), and then washed well with distilled water, until no more stain runs off. This makes a mount that can be kept for several months and is useful in making comparative studies of various growth stages of the fungus in soil. However, the acid-fuchsin-stained soil-water mounts are much superior to these semipermanent mounts for examination of the structure and content of the fungus material. Also the visibility is better when the smear does not contain the dried-down agar.

When field soil artificially infested with the fungus was studied with this technique, the germination of the chlamydospores was followed and it was proved that they were of the same fungus added to the soil.

In moist unsterilized field soil macroconidia form chlamydospores.—In a series of experiments 10 different clones of the fungus were studied in relation to their behavior in soil. When macroconidia are added to moistened field soil some germinate and form a short germ tube, but others remain ungerminated for several weeks until finally the cells round up and the conidium is converted into a chlamydospore. The hyphae from a germinated conidium also may eventually produce one or more chlamydospores as its growth becomes checked. Little or no mutation to the mycelial (M) type was observed in the process of the conversion of macroconidia to chlamydospores in untreated live soil.

In moist sterilized soil macroconidia produce much mycelium that later produces chlamydospores and mycelial (M-type) mutants.—When macroconidia are added to moist sterilized soil they germinate and the resulting hyphae may grow through the soil, branch-

ing and invading soil that has not been inoculated. The fungus may produce aerial mycelium and sporulate if it reaches the soil surface. After extensive growth or if the soil is allowed to dry slowly this mycelium dies in sections, becomes concentrated in others, and gradually forms chlamydospores. The period between development of the robust, rather extensive mycelium through the soil, and the formation of definite, mature chlamydospores may be days or weeks. Sometimes there appears to be little live mycelium left, and yet chlamydospores later appear in small sections of hyphae that are well buried in organic matter or surrounded by clay particles.

If growth of the Fusarium through the soil has been extensive, as often happens in sterilized soil, the sporodochial clone that had been added to the soil may mutate to mycelial types in the same manner as it does in artificial culture. Also, the same odors that occur in laboratory cultures may be detected in colonized soil. The mutation to the mycelial or nonsporulating (M) type was noted especially when small cans were filled with moist sterilized soil and kept loosely sealed out-of-doors. The fungus grew through the soil prolifically. As the soil dried down gradually, chlamydospores were formed. In plating out these chlamydospores, it was found that 20–60% of them were mycelial-type mutants.

Only sporodochial clones found in unsterilized field soil and in lesions on field-grown bean plants.—Several naturally infested field soils have been collected at intervals during the past 2 years and plated the same or the following day directly on special media. A modified Martin's medium (4), the Parmeter-Hood medium (3), and acidulated PDA containing added antibiotics have been used in these studies. During this period, over 3700 colonies of *F. solani* f. *phaseoli* have been recognized on these direct platings of field soil. All colonies, however, were clones of sporodochial types, and although the familiar mycelial mutants were sought and shown experimentally to grow and be recognizable on these media, none was found. The conclusion is inescapable—mycelial mutants of *F. solani* f. *phaseoli*, if they do occur and survive in field soil, must be extremely rare in the California fields studied.

In other studies, naturally infected bean plants growing in several commercial bean fields were collected, and the individual young lesions were cultured. About 350 cultures obtained from 3- to 4-week-old plants, all proved to be sporodochial clones. Since no mycelial types were recovered from young lesions, one is led to conclude further that infections in the field are rarely incited by mycelial-type mutant clones. The fields in question have been producing beans for 2–3 decades, in 1-, 2-, 3-, and 4-year rotations with various vegetable and cereal crops, so that ample time and cropping histories had been available to ensure the build-up of mycelial mutants if they formed and had survival value. Though mycelial (M) types have been demonstrated to be somewhat less pathogenic than sporodochial types in greenhouse pathogenicity tests, still, if there were mycelial types existing to any extent in field soil, one would expect to pick up an occasional mycelial type from a lesion on the bean, or in cultures made directly from field soil.

Since the organism is capable of mutating when it grows through sterilized soil, we can conclude that, first, little growth takes place through field soil, and second, if mutation does take place in growth through the bean stem before the chlamydospores are formed, either these mutants do not form chlamydospores in the bean tissue or those that are formed have low survival value under field conditions. This is in direct variance with the conclusions drawn by Maloy (2) for soils in New York.

Identification of chlamydospores in field soil.—To investigate whether *F. solani* f. *phaseoli* exists in field soil in the form of chlamydospores, collections of soil from bean fields were made when in fallow, and when in beans or in a rotation crop, and cultured on modified Martin's agar, by placing a slurry of about .01 g of the field soil on the surface of the agar.

By isolating hyphal tips of young colonies appearing 30–48 hours after placing the soil dilutions on the plates, the fungus structure from which the colony originated was identified. When the hyphae were coming out of a piece of tissue or other organic matter after a hyphal tip was removed for culture identification, the rest of the colony including the small piece of tissue was transferred from the plate to a microscope slide with a needle. It is usually necessary to crush the material a bit under a cover slip to see the fungus structure from which the young hyphae are arising. The Fusarium colonies isolated by this technique were found to arise from chlamydospores that, in turn, were associated with or imbedded in a piece of tissue or humus (Fig. 1). It was noted that germination of chlamydospores often took place at the point of attachment of the original hypha. The chlamydospore wall appeared to be thinnest at this point. *F. solani*, *f. roseum* (both carmine and noncarmine types), and *F. oxysporum* have also been found this way in natural field soil. These findings are in keeping with those of Warcup (5), who saw Fusaria arising from humus particles.

Thus, we have established that *F. solani* f. *phaseoli* occurs as chlamydospores in both naturally infested field soil and in sterilized field and virgin soils that are seeded with conidia of the organisms. Although the chlamydospores in artificially inoculated soil are often surrounded by clay particles or clinging to pieces of mica or organic matter, they are much more easily observable than those naturally occurring in field soil. These are usually deeply imbedded in disintegrating tissue and may be rather few per unit of soil.

Chlamydospores in field soil originate on and within dead cortical tissue of infected bean plants.—Since chlamydospores in field soil are usually associated with disintegrating plant tissue, a reasonable hypothesis seems to be that diseased plants grown in such soils are the source of these chlamydospores. Burkholder

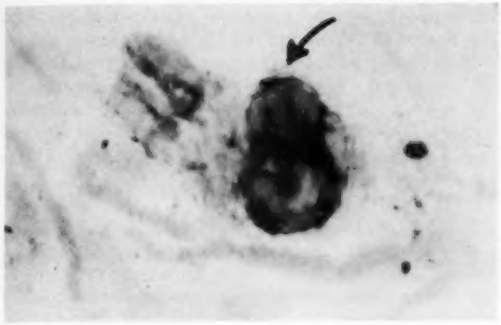

Fig. 1.—Double chlamydospore of *F. solani* f. *phaseoli* found in a piece of disintegrating tissue in naturally infested bean field soil. Surrounding hyphae attached at top of the small chlamydospore were (see arrow) later broken off by repeated tamping of the cover slip to expose entire spore. Germination of these soil-borne spores is obtained by plating on modified Martin's or some other suitable medium. A hyphal tip from this can be taken after 30–48 hours to prove structure. It is noted that often subsequent germination of dormant chlamydospores takes place at the point of attachment (see arrow) of the original hypha. The chlamydospore wall appears to be thinnest at this point.

(1) reported in 1919 that the fungus produces chlamydospores within the cortical tissue of infected bean roots.

To investigate this problem further and substantiate the above hypothesis, naturally infected small white bean plants were collected at the time of crop harvest, thoroughly washed, hand-sectioned, and stained with acid fuchsin. In addition, small white bean and Pinto plants were grown in the greenhouse, either in naturally infested field soil or in soil or sand sterilized and reinfested with a suspension of macroconidia, and the diseased parts fixed in formalin–acetic-acid–alcohol in paraffin, sectioned (7–15 μ thick), and stained with phloxine–light green.

In both transverse and longitudinal free-hand and microtome sections of field-infected plants, and in greenhouse plants from all treatments, the fungus produced abundant chlamydospores at the completion of its vegetative cycle in the necrotic tissues of the plant, both on the surface of its host and within the cortical tissue. These chlamydospores were of 2 types. Those growing on the surface of the roots were subglobular in shape and were borne mostly in chains of 2, sometimes 3. Single chlamydospores occurred occasionally. Single chlamydospores range from 9.7$\times$ 8.0 μ to 14.2$\times$14.2 μ, averaging 11.8$\times$10.5 μ. Those in chains range from 6.2–$\times$8.9 μ to 14.2$\times$14.2 μ, averaging 10.0$\times$11.8 μ. They had thick, warty walls, were filled with oil droplets, and took the stain very strongly. Others, lying mostly in the intercellular spaces and usually aligned along the long axis of the cells, were abundant in the outer cortical layers. They were rather oval or pear-shaped, and were borne in pairs, singly, or in chains of 3 or more. Single ones ranged from 6.2$\times$4.4 μ to 8.9$\times$7.1 μ, averaging 7.8$\times$5.7 μ (Fig. 2). Their walls were smooth and much thinner than those produced on the surfaces of the host. These small chlamydospores do not take the stain strongly.

Chlamydospores were not observed in the deeper cortical layers or in any other tissues, although at this stage of tissue disorganization hyphae of the fungus were seen in all tissues, even in the xylem elements.

Proof that the chlamydospores observed were those of *F. solani* f. *phaseoli* was established by macerating some of the tissue in a Waring blender and flooding plates of modified Martin's medium with dilutions of the resulting suspension. As the chlamydospores of different types and sizes were observed to germinate under the compound microscope, they were individually removed and planted on another agar plate. In this manner it was possible to demonstrate by cultural characters, directly and without doubt, that the chlamydospores observed in and on the diseased bean tissue were in fact *F. solani* f. *phaseoli*. Further confirmation was made by pathogenicity tests of representative clones so isolated.

Chlamydospores formed only on lesions on senescent plants.—The stage of the host plant at which the chlamydospores were formed was determined by studying sections prepared at intervals from the growing infected plants. Only at the end of the plant's life, and in the necrotic tissues, were chlamydospores formed. Exhaustion of the food material in the host tissues by the fungus seems to be the prerequisite for formation of these resting structures. This theory was substantiated by their great abundance in disorganized, dying tissues from plants killed early in their life by heavy disease development. Conidia were observed, however, on the surface of infected hypocotyls of both field- and greenhouse-grown bean plants, on lesions that had reached the surface of the soil, and where moisture, light, and, apparently, nutrients were adequate.

Discussion.—That the fungus *F. solani* f. *phaseoli*, or other species of Fusarium, may occur in soil in the form of chlamydospores is not a new idea. Burk-

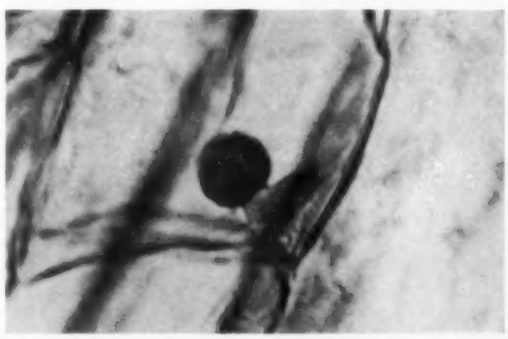

Fig. 2.—Chlamydospore of *F. solani* f. *phaseoli* in an intercellular space in the outer cortex of infected hypocotyl of Pinto bean grown in artificially infested soil in the greenhouse. Free-hand section stained with acid fuchsin.

holder presupposed this in 1919 (1) on the basis that the fungus produced them in pure culture. Warcup (5) established beyond any doubt that Fusarium species exist in the soil as chlamydospores by isolating and growing them in pure culture. However, visual proof that the chlamydospores of a pathogenic Fusarium do occur in naturally infested field soil for the most part has been lacking.

Evidence presented here for the Fusarium root and stem rot of bean seems to show conclusively that the pathogen *F. solani* f. *phaseoli* in naturally infested field soil exists exclusively, or almost so, in the form of chlamydospores. The exception to this is the presence of conidia, principally macroconidia, which may be formed under certain conditions on affected parts of the plant that are exposed to light, and which may be washed into the soil or reach the soil during harvesting or cultivation. It seems likely that such conidia, to the extent that they survive in soil, do so by converting directly or indirectly through germination into chlamydospores. Infection in the field, then, would result principally from the germination of chlamydospores.

Evidence is also presented that the fungus does not normally grow saprophytically through field soil searching for a host plant. Rather the bean plant must send its underground parts through the soil to reach the chlamydospores of the fungus before a pathological relationship may be established. Dr. D. Burke has expressed a similar view (personal communication).

The pathogen is shown to produce large numbers of chlamydospores in the necrotic tissues of the host, these being of different morphological and physiological age, apparently with different capacities for survival. The chlamydospores are released into the soil with the disintegration of the diseased host tissue, since the chlamydospores of the fungus found in the soil of a fallowed field are generally imbedded in particles of organic matter.

Furthermore, although there are many sporodochial clones of *F. solani* f. *phaseoli* that can be isolated from both the plant and the soil, the mycelial types have not been found in nature in California as reported in New York by Maloy. This is not surprising, in view of the fact that mycelial clones are much weaker pathogens and would probably have lower survival value than the common sporodochial types. Since the mycelium has not been observed to grow through field soil it is not likely that mutation from the sporodochial to mycelial types is as frequent in nature in the absence of its host as it is in the laboratory culture tube, nor as has been observed when the mycelium grows through sterilized soil in small, closed containers.

All indications obtained from the isolation of single, young lesions on field-grown bean plants and isolations of the fungus directly from soil show that the surviving clones of *F. solani* f. *phaseoli* are sporodochial types. Mycelial mutants of low pathogenicity have not been isolated from field soils.

The role of the conidia of *F. solani* f. *phaseoli*, then, appears to be principally that of distributing the fungus (with water, soil, or plant parts), and of replenishing the chlamydospore inoculum in the soil by conversion. The chlamydospore then emerges as the major soil-borne propagule of the pathogen, principally responsible for both survival in soil and primary infection of the subterranean parts of the bean, in the field.—University of California, Berkeley.

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BOOK LISTINGS

Wirusologia Ogolna (General Virology).—*E. Mikulaszek and W. T. Dobrzanski*, eds.—1960. In Polish. Government Scientific Publ. Office, Warsaw. 580 p.

This Polish textbook of virology brings together not only viruses that cause diseases in humans, animals, plants, bacteria, and insects, but also the work, literature, and viewpoints of eastern and western virologists. It is unfortunate that it can be read only by those who understand Polish. There are 13 chapters, by 7 authors, covering the nature of viruses, their structure, methods of isolation and purification, electron microscopy, mutations, genetics, virus-cell interrelationships, metabolic changes in host cells, lysogeny, immunochemistry, serology, and ecological problems. Although plant viruses are partly integrated into the first 12 chapters, the last and longest (90 pages), by A. Kozlowska, deals with plant viruses only. The author discusses symptomatology, transmission, spread, serology, multiplication, purification, morphology, inactivation, mutation, acquired immunity, and plant protection. The chapter by H. Makower on virus mutation mentions the Michurin-Lamarck ideas, still held in Soviet Russia, but then deals with classical genetics only. This chapter cites the work of Bawden, Price, J. H. Jensen, McKinney, Thung, Kunkel, Johnson, Stanley, Valleau, and many others. The chapter on virus genetics, by A. Kozinski, is based exclusively on work done with bacterial viruses in the United States, and on Burnett's work in Australia. In the preface to the volume the editors mention with regret that Dr. Kozinski, in the meantime, had left (he is now on the staff of Johns Hopkins University).

DIURNAL SPORE RELEASE OF PIRICULARIA ORYZAE FROM RICE LEAVES

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SUMMARY

When lesions of *Piricularia oryzae* on attached leaves of the rice varieties Colusa, LaCrosse, C.I. 8970, or Arkrose were kept in 100% RH and 70–80°F, conidia were released only at night. Lesions kept continuously in high RH produced twice to several hundred times as many spores as did lesions so kept at night but allowed to dry during the day. A method was devised of following spore release over a 24-hour period. Use of this revolving Petri dish spore meter showed that lesions kept moist began release soon after dark, reached a maximum within a few hours, and slowly decreased until dawn, when release ceased. For lesions given artificial dew periods of less than 24 hours, release of the fresh crop of spores began 6–8 hours after the start of the period and stopped soon after dawn; a few spores formed the previous night were sometimes released at the start of a dew period.

Reports from abroad (5, 13) and from this laboratory (14) indicate that conidia of *Piricularia oryzae* Cav. are found in the air over rice paddies at night, especially from midnight until shortly after sunrise. Since it is known that sporulation on lesions occurs only when conditions approach 100% relative humidity (RH) within a favorable temperature range (5, 6) it might be assumed that spores are found at night because that is when RH is 100% and dew lasts long enough—5–8 hours—for spores to be produced. Reports on some other fungi show a diurnal periodicity of spore discharge (1, 2, 8, 9, 10, 11, 12, 16, 17) or at least of spores in the atmosphere (3, 4, 7, 14). This raises the question of whether such a diurnal periodicity in *P. oryzae* is entirely a function of the dew period or whether light may also be involved. Without presenting data, Hashioka (5) stated that dissemination is delayed in the day even when RH is high.

For studying the possibility that a light-controlled diurnal cycle exists, methods were chosen in which the numbers of spores released from individual lesions could be determined. In selecting these methods, thought was given to the requirements of high RH for sporulation, and the desirability of leaving the leaf attached to the plant so that lesions could enlarge naturally and sporulation be studied over a period of days.

Materials and methods.—All the work reported was done in the greenhouse or in a plant growth room. Rice varieties Colusa, LaCrosse, and C.I. 8970 were seeded in 4-in. black plastic pots containing a fertile clay loam. The seedlings were thinned to 5 per pot. When the plants were about a month old they were inoculated by spraying them with a dilute water suspension of an isolate (No. 770) obtained from Dr. F. M. Latterell, of these laboratories. The inoculated plants were placed in dew cabinets maintained at 72°F for 16 hours. One week later, lesions were selected for study.

Two procedures were used in collecting spores released from sporulating lesions. In the first, a leaf of one of the rice plants that bore discrete lesions was threaded through a 15-cm Petri dish without detaching it from the plant. The rim of the bottom of the dish had been built up in several places with masking tape in order to support the lid about 2 mm above the rim and thus prevent crushing the leaf tissue. A piece of moist filter paper was placed in the bottom of the dish, and the lid of a small 6-cm dish partially filled with 2% water agar was placed on the filter paper. Any spores formed on the underside of the lesion could fall onto the surface of the agar, where they could be counted easily.

In the second procedure, an apparatus with which to study the time of spore release was assembled as follows: a 24-hour clock (of the type used in a Taylor dew meter) (15) was firmly attached to a plywood base; a 2.5-cm hole was formed in the bottom of a Petri dish (15 cm diameter), and the dish was supported so the rotating shaft of the clock came up slightly through the hole; a ring of moistened filter paper was placed around the hole in the bottom of the dish; a one-holed rubber stopper of slightly less diameter than the hole was placed on the shaft of the clock so as to support the lid of a 10-cm Petri dish partially filled with 2% water agar.

This agar plate rotated once in 24 hours and did not touch the top or bottom of the larger, stationary Petri dish (moist chamber) in which it was enclosed. This apparatus is hereinafter called a spore meter.

A leaf bearing a discrete lesion was sprayed with a fine mist of tap water until coated with a "dew." The leaf was placed across the larger dish with the lesion near the edge of the agar plate, and the lid put in place. As the plate revolved, any spores released fell onto the agar and formed an arc-shaped deposit. At the start of a test, the agar plate was marked with a wax pencil opposite the lesion, and at the end of the test this mark was used as the reference for marking a 24-hour grid of 24 radii on the agar with a razor blade. Total spore counts for each hour could be determined by counting all the spores deposited between two lines of the grid, or a sample count could be made by counting a strip 1 mm wide along such a radius.

Results.—The first procedure described above was used to study the effect on spore production of drying during the day as opposed to keeping a lesion at a high RH for 24 hours. In a typical test, C.I. 8970 and

LaCrosse plants were inoculated, and two weeks later discrete lesions were enclosed in Petri dishes at 1:30 P.M. The following morning at 8 A.M. some leaves were removed from the dishes and allowed to dry while fresh agar plates were placed under the leaves in all dishes, and the leaves that had been dry during the day were again placed in their Petri-dish moist chambers. The experiment was conducted in a laboratory at 80°F near a north window, where the plants were in light daily from about 7:30 A.M. to 4:30 P.M.

Many more spores were deposited on the agar plates when lesions were kept continuously in a moist chamber than when lesions were alternately wet and dried (Table 1). The time of release is also noteworthy; for those lesions kept continuously in a high RH, spores were released from their conidiophores almost entirely at night, although during the day the lesions were observed to be covered with spore-bearing conidiophores.

In these experiments, the spores appeared to drop vertically onto the agar, with very little drift. The first deposition covered a circular or oval area, whereas deposits on subsequent nights were typically ovals with vacant centers, i.e., sporulation on the lesion was heaviest in a zone near the margin, and as the lesion expanded it ceased to support sporulation at its center.

Fig. 1 shows a series of camera lucida drawings of the spore deposits from 7 lesions, 3 on Colusa and 4 on C.I. 8970, made daily for 1 week. The experiment was set up at 10 A.M. on the eighth day after inoculation. The lesions were kept in a humid atmosphere in a shaded portion (300 ft-c) of a greenhouse maintained at 80°F (±5°). Agar plates were changed once a day in order to count spores and make drawings of the deposit. The largest numbers of spores were deposited during the second evening; the number deposited in succeeding days tended to level out although the lesions kept enlarging.

Table 2 shows the results when lesions on either C.I. 8970 or LaCrosse were placed in the spore meter for 3–4 days. The study of a lesion on C.I. 8970 was made in a laboratory maintained at 80°F and receiving daylight from 7:30 A.M. to 4:30 P.M. (approx. 100 ft-c).

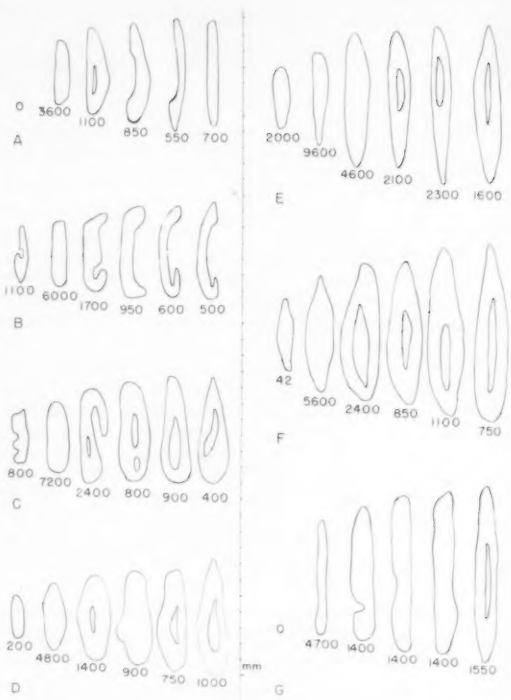

Fig. 1.—Camera lucida drawings of the outline of spore deposits on agar plates of conidia of *Piricularia oryzae* from rice leaves. Lesions were kept continuously in an atmosphere of 100% RH at about 80°F. Agar plates were changed and drawings made at daily intervals except that the fourth drawing from the left in each series represents a 2-day deposit. **A–C)** From lesions on Colusa; **D–G)** from lesions on C.I. 8970. Numbers under each drawing indicate number of spores in each deposit.

The two lesions on LaCrosse were placed in spore meters in a constant-temperature room (72°F) with fluorescent lights from 6 A.M. to 6 P.M. (approx. 200 ft-c). The agar plates were changed daily at 8 A.M. Spore counts were made at hourly intervals by counting the number of spores in a 1-mm-wide strip along a

Table 1.—The number of spores[a] deposited on agar plates from the under surface of lesions of *P. oryzae* on the rice varieties LaCrosse or C.I. 8970; separate lesions were kept in about 100% RH for 24 hours a day or for the night hours only.

Variety	Time kept at 100% RH	Day 1 1:30 P.M. to 8 P.M.	Day 2 8 A.M. to 4 P.M.	Day 2 4 P.M. to 8 P.M.	Day 3 8 A.M. to 4 P.M.	Day 3 4 P.M. to 8 P.M.	Total
LaCrosse							
Lesion 1	24-hr day	8	0	4000	0	2400	6408
Lesion 2	24-hr day	0	0	832	0	1600	2423
Lesion 3	night only	5		273		340	618
Lesion 4	night only	57		170		5	232
C.I. 8970							
Lesion 1	24-hr day	60	0	8400	23	1100	9583
Lesion 2	24-hr day	0	0	12000	11	1500	13511
Lesion 3	night only	14		4000		2200	6214

[a] Numbers above estimated to the nearest 100; zero indicates no spores deposited; blanks indicate no observation.

Table 2.—Number of spores deposited on revolving Petri-dish spore meters during 12-minute intervals at time indicated from lesions enclosed in 100% RH.

Time	C.I. 8970—lesion no. 1			LaCrosse—lesion no. 1				LaCrosse—lesion no. 2		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3
8 A.M.	(light	0	3	(light	0	0	4	(light	0	0
9	from	0	0	from	0	3	0	from	0	4
10	7:30 A.M.	0	0	6 A.M.	0	1	0	6 A.M.	0	0
11	to	0	0	to	0	0	0	to	0	0
12	4:30 P.M.)	0	0	6 P.M.)	0	0	0	6 P.M.)	0	0
1 P.M.		0	0		0	0	0		0	0
2	start	0	0		0	0	0		0	0
3	0	0	0		0	0	0		0	0
4	0	0	0		0	0	0		0	0
5	0	0	1	start	0	0	0	start	0	0
6	0	18	0	0	3	4	3	0	0	0
7	0	292	0	0	18	8	20	0	0	13
8	0	354	1	0	50	22	25	0	3	10
9	0	390	73	0	78	36	45	0	60	31
10	0	319	147	0	65	32	85	0	87	73
11	0	278	163	0	87	54	79	0	101	72
12	0	261	114	0	81	75	121	0	118	78
1 A.M.	0	172	92	0	69	72	80	0	108	84
2	0	138	110	0	52	64	88	0	93	79
3	0	102	78	0	50	84	96	0	104	66
4	0	99	53	0	46	54	96	0	91	55
5	0	84	55	0	24	60	45	0	91	73
6	0	33	10	0	4	8	10	0	90	18
7	0	6	1	0	0	1	1	0	7	1

radius of the arc of deposit. Deposition began almost as soon as darkness fell, rose to a maximum within a few hours, and gradually diminished until dawn, when it ceased.

Since the distance between hourly radii in the area of the spore deposit was 5–6 mm it is possible to make an estimate of the total number of spores deposited during each night (a 1-mm-wide strip would represent a deposit taking place during about 12 minutes). Such an estimate for data in Table 2 yields 15,000 and 6000 spores for the second and third evenings from the lesion on C.I. 8970; 3100, 2700, and 4000 spores for the second, third, and fourth evenings from lesion no. 1 in LaCrosse; and 4800 and 3200 spores for the second and third evenings from lesion no. 2 on LaCrosse.

The spore meter can also be used to mimic conditions found in nature, i.e., to see how different periods of dew affect the numbers of spores released and when they are released. Since dew in the field does not evaporate until 2 or 3 hours after sunrise, spore meters were set up to run for periods of 14, 12, and 10 hours, with the last 2 hours of each dew period in the light. The test was conducted in a room main-

Table 3.—No. of spores released during 12-minute intervals at the hour indicated from lesions of *Piricularia oryzae* kept in spore meters for 10, 12, or 14 hours for 2 days and for 15 hours on the third day.

Time	10-hour dew period			12-hour dew period			14-hour dew period		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
5 P.M.			1			0			0
6[a]			3			1	0	0	0
7			1			3	0	0	14
8			0	0	0	1	0	0	0
9			0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	10	0	0	3	0	0	0
12	0	0	3	0	0	8	0	9	0
1 A.M.	0	0	33	0	0	23	0	18	6
2	0	0	61	0	2	32	0	105	24
3	0	0	120	0	6	58	0	184	30
4	0	0	89	8	9	67	2	183	43
5	1	1	53	13	32	67	15	218	41
6[b]	4	4	5	4	2	4	3	193	35
7	2	1	2	5	0	0	4	41	17
8	0	0	0	0	0	0	0	8	1

[a] Lights turned off.
[b] Lights turned on.

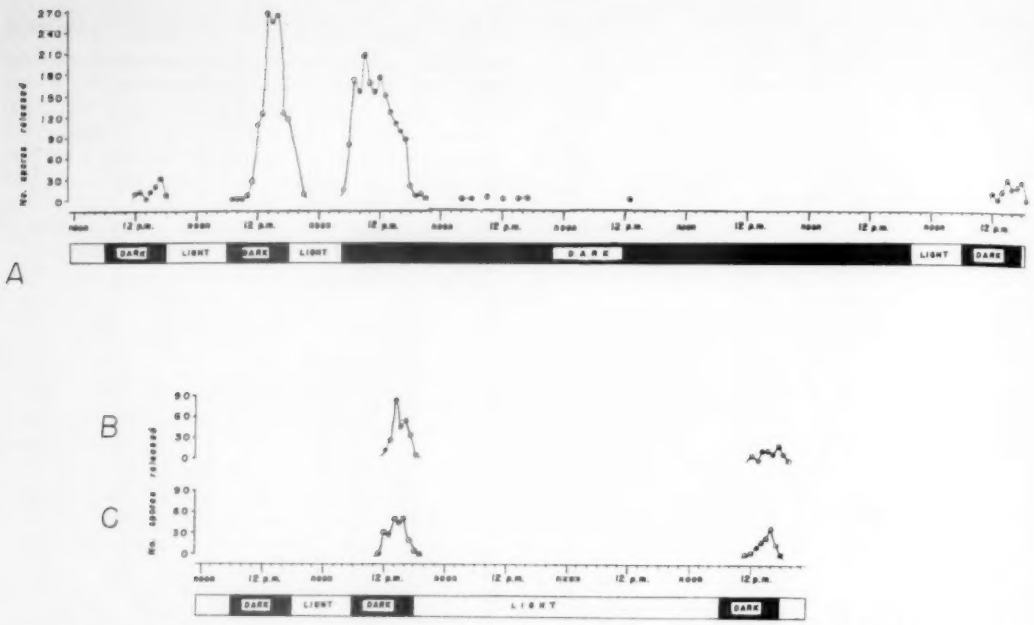

Fig. 2.—The effect of a prolonged period of either light or darkness on spore release from lesions of *Piricularia oryzae* on rice leaves. Lesions were kept continuously in 100% RH, and spores were collected on the surface of a revolving Petri dish spore meter. Numbers of spores were counted in a strip along a radius of the arc of deposit and represent a 12-minute deposit centered at the hour indicated. **A)** The effect of prolonged darkness on spore release from a lesion on Arkrose. **B** and **C)** The effect of prolonged light on spore release from 2 lesions on Colusa.

tained at 70°F. One-week-old lesions on C.I. 8970 were sprayed with water until a moderate "dew" had been deposited and were then placed in spore meters 0, 2, or 4 hours after the beginning of a night lasting 12 hours. They all were removed and allowed to dry 2 hours after the lights (fluorescent; 200 ft-c) were turned on. Lesions were returned to spore meters on the same schedule for the following night, but all lesions were given a 15-hour dew period on the third night.

The numbers of these tests were not sufficient to enable one to predict what a group of lesions in the field might do on a night in a given dew period. The results, however, do seem to fit a pattern, and data considered representative for each treatment are presented in Table 3. A few spores are sometimes released within the first 3 hours after a lesion is placed in 100% RH, but the fresh crop of spores is released starting about the eighth hour. The presence of light tends to stop their release, or at least to slow it down.

Having established a diurnal cycle for spore release regulated by light as well as humidity during a normal 24-hour day, we thought it would be interesting to find whether the cycle could be delayed by extended periods of light or prolonged in darkness. For this purpose, lesions were maintained in spore meters until a diurnal cycle was established; then some were kept in continuous darkness and others in continuous light for several days, following which they were returned to a more normal daily regime of 12 hours of light and 12 hours of darkness. Again, the experiment was performed in a room maintained at 70°F. Agar plates were replaced at 11 A.M. daily.

The data in Fig. 2 show a cessation of spore release from one lesion after 2 days of continued darkness, and a resumption of a normal cycle of release after subsequent exposure to a light period of 10 hours. Two lesions exposed to 2 days of continuous light after the establishment of a normal diurnal cycle did not release spores, although they were covered with spore-bearing conidiophores, until returned to darkness.

Discussion.—Both procedures seem to satisfy the requirements for maintaining high RH and for permitting the leaf to remain attached to the plant and the lesion undisturbed. They are not, however, satisfactory for estimating the total number of spores produced on a single lesion, since lesions typically sporulate on both upper and lower surfaces and our procedures permitted collection of spores from only one surface at a time.

As far as the authors are aware, the actual mechanism of release has not been reported, and we failed in our attempts to observe "take off." It may be that a method will have to be devised for seeing "take off" in the dark, at least for seeing it in any quantity. Until then, it would seem best to use the term "release" instead of terms such as "abscission" or "dis-

charge," which imply that the mechanism of "take off" is known.—Biological Laboratories, Fort Detrick, Frederick, Maryland.

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RELATION OF PETROLEUM OIL COMPOSITION TO PHYTOTOXICITY AND SIGATOKA DISEASE CONTROL ON BANANA LEAVES

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SUMMARY

The saturated, aromatic, and nonhydrocarbon fractions from heavy paraffinic, light paraffinic, heavy naphthenic, and light naphthenic oils were sprayed 4 times at 2-week intervals on banana leaves at low-volume deposit rates not exceeding 2.1 mg of oil/100 cm^2 of leaf surface. The saturated fractions from the first 3 oils caused phytotoxic leaf flecking and gave Sigatoka disease control, while the saturated fraction from the light naphthenic oil caused almost no flecking but gave good disease control. The aromatic and nonhydrocarbon fractions from the 4 oils caused little or no leaf flecking and gave no appreciable control of the disease. When a saturated and an aromatic fraction were compared at deposit rates of 2.1, 4.2, 8.4, and 16.8 mg/100 cm^2 respectively, the saturated fraction caused considerable flecking at all deposit levels while the aromatic did so only at the 3 higher rates. At the first 3 rates the saturated fraction gave better disease control while at 16.8 mg both fractions gave about equal control. It is suggested that the development of improved spray oils for bananas requires further studies using known low-volume deposit rates and giving emphasis to the testing of saturated fractions from light naphthenic oils.

Petroleum oil-mist sprays at 1–3 gal./A. are widely used to control the Sigatoka leaf-spot disease of bananas caused by *Mycosphaerella musicola* Leach (4, 9). Superior disease control and ease of application are important advantages of low-volume oil sprays over conventional high-volume Bordeaux applications (6, 7, 12). One disadvantage of oil sprays, however, is their tendency to cause phytotoxicity in the form of leaf flecking and lower fruit yields (1, 10, 11). There is a need therefore, to develop oils that cause little or no phytotoxicity and maintain good Sigatoka control. In this initial study, 4 types of oils and their fractions differing in chemical composition were tested to determine: 1) the fraction(s) that causes phytotoxicity and gives Sigatoka disease control for each oil type, 2) whether certain oil types are superior to others for spraying bananas, and 3) the effect of certain fractions on phytotoxicity and disease control at different spray deposit levels.

Materials and Methods.—Four relatively unrefined petroleum oils were used that differed in composition (paraffinic or naphthenic) and in molecular weight

(heavy or light). A naphthenic oil, in contrast to a paraffinic oil, has more molecules containing saturated ring structures and complex chain branching (Unpublished data from the Esso Research and Engineering Co.). Heavy oils are characterized by larger molecules and are more viscous and less volatile than light oils. The 4 oil types used in this experiment were: heavy paraffinic, light paraffinic, heavy naphthenic, and light naphthenic.

A portion of each of the 4 oils was divided by silica gel chromatography into the following chemically different fractions: saturated, aromatic, and nonhydrocarbon. The saturated fraction from each oil type contains both paraffins and naphthenes which have no unsaturated chemical bonds. Paraffins are either straight or branched chain hydrocarbon molecules. Naphthenes are cyclic hydrocarbon molecules. The aromatic fraction from each oil consists of hydrocarbon molecules characterized by the presence of 1 or more benzene rings. The molecules of the so-called nonhydrocarbon fraction, in addition to carbon and hydrogen, contain 1 or more other elements mainly: oxygen, nitrogen, and sulfur. Quantitative characteristics of the 4 whole oils and their fractions are presented in Tables 1 and 2.

Table 1.—Quantitative characteristics of the 4 whole oils.

	Whole oils			
	Paraffinic		Naphthenic	
Characteristics	Heavy	Light	Heavy	Light
Viscosity, SUS at 100°F.	280	112	1169	82
Molecular weight[a]	435	335	412	296
Distillation, °F.[b]				
Initial	688	621	735	572
10%	757	689	774	618
50%	849	728	820	661
90%	Cracked	767	888	720
Complete		816	938	764
Pour point, °F.	103[c]	−5	−5	−20
Unsulfonated residue	80	82	74	77
Viscosity index	95	67	16	27
Composition (Silica gel)				
Saturates, %	69.7	71.2	56.0	65.9
Aromatics, %	28.0	28.0	39.0	32.7
Nonhydrocarbons, %	2.3	0.8	5.0	1.4
Composition of saturated fraction[d]				
Paraffins	32.2	27.2	6.0	6.5
Naphthenes	67.8	72.8	94.0	93.5
Peroxide number[e]	20.1	0.3	13.0	0.2
Neutralization number[f]	0.45	0.11	1.57	0.70
Specific gravity, 60°/60°F.	0.891	0.888	0.928	0.904
Refractive index, 20°C.	1.477	1.492	1.493	1.497

[a] Obtained by cryoscopy.
[b] Converted to 760 mm Hg from 10 mm data.
[c] Can be sprayed at room temperatures.
[d] Obtained by mass spectrometry.
[e] me of peroxide/L of oil.
[f] mg of KOH/g of oil.

Three tests were carried out in which all or some of the above materials were evaluated for phytotoxicity and disease control. In the first test, each whole oil and its 3 fractions were used. The whole oil was sprayed alone: its fractions were applied alone or in the presence of another fraction or in a carrier as follows: 1) saturated and aromatic fractions alone, 2) blended together, 3) blended separately with nonhydrocarbon, 4) each of the 3 fractions diluted with a very light carrier oil (a paraffinic oil, viscosity 32 SUS at 100°F., distillation range 402–477°F. at atmospheric pressure, unsulfonated residue 97%), which in previous tests produced no phytotoxicity and gave no Sigatoka control. The whole oils and fractions were applied by a precision spray technique described previously (2). Each whole oil was deposited at the rate of 2.1 mg/100 cm^2 of leaf area. An equivalent deposit is produced at 1.5 gal./A. by a commercial knapsack mist blower (2). The amount of each fraction deposited was equal to the amount of that fraction in the whole oil deposit. In the second test, the 4 whole oils and their respective saturated fractions were used. The spray materials were applied at 2.1 mg/100 cm^2. In the third test, the saturated fraction and the aromatic fraction from the heavy paraffinic whole oil were tested at 4 deposit rates, i.e. 2.1, 4.2, 8.4, and 16.8 mg/100 cm^2.

The first application of each spray material was made on the upper surface of the youngest unfurled leaf on Dwarf Cavendish banana plants. During a test, each leaf received a total of 4 sprays at 2-week intervals which approximates commercial spray prac-

Table 2.—Quantitative characteristics of the saturated, aromatic, and nonhydrocarbon fractions from the 4 whole oils.

	Fractions											
	Heavy paraffinic			Light paraffinic			Heavy naphthenic			Light naphthenic		
Characteristics	Satur.	Arom.	Nonhyd.	Satur.	Arom.	Nonhyd.	Satur.	Arom.	Nonhyd.	Satur.	Arom.	Nonhyd.
Viscosity, SUS at 100°F.	135	1475	SS[a]	85	641		366	7860	SS[a]	70	148	1043
Molecular weight[b]	451	395	419	390		350	446	400	390	308	262	341
Specific gravity[c]	0.82	0.97	1.01	0.83		1.10	0.87	0.98	1.01	0.87	0.98	1.02
Refractive index[d]	1.45	1.53	1.54	1.46	1.55	1.49	1.47	1.53	1.54	1.47	1.55	1.51
Neutralization No.[e]	0.06	0.13		0.06	0.41		0.17	0.09		0.14	0.04	

[a] Semisolid.
[b] Obtained by cryoscopy.
[c] 60°/60°F.
[d] 20°C.
[e] mg KOH/g of oil.

Table 3.—Evaluation of 4 types of oils and their fractions for phytotoxic flecking and effect on Sigatoka disease incidence on banana leaves sprayed 4 times at 2-week intervals[a].

Fractions	Phytotoxic flecking[b]				Disease incidence[c]			
	Paraffinic type		Naphthenic type		Paraffinic type		Naphthenic type	
	Heavy[d]	Light[d]	Heavy[d]	Light[e]	Heavy[d]	Light[d]	Heavy[d]	Light[d]
Whole oil	39	20	19	4	0.6	0.3	0.4	0.2
Saturated	29	34	21	1	0.4	0.2	0.2	0.2
Saturated + aromatic	31	26	8	1	0.2	0.3	0.5	0.3
Saturated + nonhydrocarbon	26	32	13	1	0.3	0.2	0.2	0.1
Saturated + carrier oil[f]	17	23	7	0	0.4	0.2	0.4	0.1
Aromatic	1	0	[g]	0	0.9	1.1	[g]	1.1
Aromatic + nonhydrocarbon	5	0	[g]	0	0.8	1.0	[g]	1.0
Aromatic + carrier oil	2	0	0	0	1.1	1.1	1.2	0.7
Nonhydrocarbon + carrier oil	1	0	1	0	1.0	1.1	1.5[h]	1.2

[a] Each number is the average value for 8 replicate leaves. Data taken 2 weeks after the final spray.
[b] Percent of sprayed leaf area with flecks.
[c] Disease incidence rating: % diseased area on sprayed leaf half divided by % diseased area on unsprayed leaf half.
[d] With this oil type, the combined data from all the sprays containing the saturated fraction differed significantly at the 1% level from the combined data of all the sprays with no saturated fraction.
[e] Significant difference at the 1% level between the whole oil and the other data.
[f] The carrier oil alone caused no flecking and gave no disease control when compared to unsprayed checks.
[g] Could not be sprayed because of very high viscosity.
[h] Significantly more disease on sprayed leaf halves than on the unsprayed ones.

tices. Preliminary trials showed that there is less error in disease evaluation when the treated and check areas are on the same leaf rather than on different leaves. On each treated leaf, therefore, the right side was sprayed while the left side was used as an unsprayed check for disease. Each spray material was applied on 8 replicate leaves. It was also shown in preliminary trials that at certain seasons one half of a leaf may have more disease than the other half. This factor was taken into account in the present tests by including entire unsprayed leaves as additional checks. The disease data in the tables were corrected for any natural variation in disease incidence between leaf halves occurring at the time of each observation.

Observations on phytotoxicity and disease incidence were made 2 weeks after the final spray. The only phytotoxic symptom observed on the treated leaves was the presence of small rust-colored flecks approximately 0.5–1.0 mm wide and 1.0–4.0 mm long. Phytotoxicity was evaluated as the percentage of sprayed leaf area covered with these flecks. Disease incidence on each leaf was evaluated as the percentage of sprayed leaf area with disease (right-half) divided by the percentage of unsprayed leaf area with disease (left-half). A low disease rating indicates low disease incidence.

Results.—*First test: Comparison of fractions within each oil type.*—The flecking and disease data for each oil type are presented in the vertical columns of Table 3. With the heavy paraffinic, light paraffinic, and heavy naphthenic types, abundant flecking was caused by the sprays containing the saturated fraction. Sprays containing no saturated fraction caused little or no flecking. Analysis of variance showed a highly significant difference in the results between the spray materials containing the saturated fraction and those that did not. With the light naphthenic oil type, on the other hand, abundant flecking did not occur. The whole oil flecked only slightly and the other light naphthenic sprays caused either a trace or no flecking.

The disease data for each oil type show that appreciable disease control occurred only with the sprays containing the saturated fraction. Sprays containing no saturated fraction gave either poor or no disease control. There was a highly significant difference in the results between the spray materials containing the saturated fraction and those that did not.

In Table 3, the figures should be compared only within each column (oil type) and not between columns. Each of the 4 oil types were tested singly at different times of the year and seasonal variations might have contributed to differences in the results between types.

Second test: Four whole oils and their saturated fractions evaluated simultaneously.—In the first test, the saturated fraction was shown to be chiefly responsible for phytotoxicity and disease control for 3 of the oil types. With the light naphthenic type, on the other hand, the sprays containing the saturated fraction caused almost no flecking but were the only ones that gave good disease control. The lack of flecking might have been due either to the nature of the spray material or to a seasonal effect. These 2 possibilities were examined in a second test in which the 4 types of saturated fractions and whole oils were evaluated simultaneously.

The results from this test showed that the light naphthenic saturated fraction and whole oil caused only 7 and 3% flecking, respectively. These figures were highly significantly less than the flecking caused by the other 3 types of saturated fractions and whole oils. Disease control was good and did not differ

significantly among the 4 saturated fractions or among the 4 whole oils.

The second test indicates that the superior performance of the light naphthenic saturated fraction in the first test was not due to a seasonal effect but was probably a characteristic of the oil type. This light naphthenic saturated fraction and the whole oil seem to be better suited as an antifungal spray oil for banana leaves than any of the other spray materials tested.

Third test: Saturated and aromatic fractions from one whole oil compared at increasing deposit rates.—The saturated and aromatic fractions are generally the major constituents of spray oils. The purpose of this test was to determine the amount of phytotoxic flecking and disease control obtained by these 2 fractions when sprayed at increasing deposit levels starting at the low-volume rate of about 2 mg/100 cm^2. The saturated and aromatic fractions from the heavy paraffinic whole oil were used as the spray materials.

The phytotoxicity results in Table 4 show that the saturated fraction even at the lowest deposit rate caused a considerable amount of flecking. As the deposit was increased, the percent of flecking leaf area increased gradually. The aromatic fraction at the lowest rate caused almost no flecking. As the deposit increased, the percent of flecking increased rapidly, and at the two highest deposit rates the aromatic fraction caused significantly more flecking than the saturated. At the low-volume deposit rate, the saturated fraction, not the aromatic, was the chief cause of phytotoxic flecking.

The disease-incidence results in Table 4 show that the saturated fraction gave good disease control at all deposit rates. The aromatic fraction gave relatively poor control at the 2 lowest rates but good control at the 2 highest rates. If the flecking results are compared when the 2 fractions are giving equal disease control, the saturated fraction caused only about one-half as much flecking as did the aromatic, indicating that the aromatic fraction would not be suitable for Sigatoka control.

Table 4.—Saturated and aromatic oil fractions tested at different deposit rates for phytotoxic flecking and effect on disease incidence on banana leaves[a].

Deposit rate	Phytotoxic flecking[b]		Disease incidence[c]	
mg/100 cm^2	Saturated	Aromatic	Saturated	Aromatic
2.1	26	4	0.09	0.77
4.2	33	34	0.05	0.38
8.4	39	52	0.01	0.09
16.8	48	61	0.00	0.01

[a] Each figure is the average for 8 replicate leaves which received 4 oil applications at 2-week intervals.

[b] Percent of sprayed leaf area with flecks. Highly significant differences between treatments with saturated and aromatic fractions except at 4.2 mg deposit.

[c] Disease incidence rating = % diseased area on sprayed leaf half divided by the % diseased area on unsprayed leaf half. Highly significant differences between treatments with saturated and aromatic fractions at 2.1 and 4.2 mg deposits.

Discussion.—Phytotoxicity was evaluated in these trials on the basis of leaf flecking. The ultimate measure of phototoxicity, of course, is not leaf flecking, but banana fruit yield. On the basis of fruit yield, however, it would take several years and many acres of plants to screen even a small number of oils. The leaf flecking criterion, although an indirect one, permits a quick 8–10 week test requiring only a small number of plants. Since these flecks are the most common phytotoxic symptoms and result from injured mesophyll cells (unpublished data) there is reason to believe that flecking is probably a reliable measure for evaluating oil spray phytotoxicity on banana plants.

The saturated fraction is the active component in the spray oils used on bananas. At the low-volume deposit rates of the first test, the saturated fraction from the 4 oil types was the only component that gave appreciable Sigatoka disease control. In the oils causing abundant flecking, the saturated fraction was the chief cause of these phytotoxic symptoms. In the third test, the saturated fraction from the heavy paraffinic oil gave good disease control and abundant flecking at all the deposit levels used. The results from the light naphthenic saturated fraction in the first test, on the other hand, showed that good disease control can be obtained without the presence of abundant flecking. It seems likely, therefore, that better spray oils for bananas may be found by testing saturated fractions from various oils.

The aromatic and nonhydrocarbon fractions are probably inert in low-volume oil sprays used on bananas. These fractions caused little or no flecking and gave no appreciable disease control at the rates used in the first test. The results with the aromatic fraction were surprising since it is generally agreed that this fraction is a primary cause of phytotoxicity in agricultural spray oils applied to other plants (3, 5, 8). The third test showed, however, that the effect of the aromatic fraction on banana leaves was influenced by the deposit rate. This fraction caused little flecking and gave little disease control at the lowest deposit rate but caused abundant phytotoxicity at about 4 mg/100 cm^2 and above, accompanied by increasing disease control. A typical spray oil with about 10% aromatic content (by silica gel chromatography) would have to be deposited at the rate of 40 mg/100 cm^2 before the aromatic fraction would achieve the 4 mg level. This deposit rate of the spray oil is high and very unlikely to occur with low-volume mist sprays (2). The first and third tests show, therefore, that at known low-volume deposit levels the aromatic fractions tested are probably not an important cause of flecking or disease control on banana leaves.

Of the 4 oil types examined, in the first and second tests, the light naphthenic was the best for spraying bananas because it caused little flecking and gave good

disease control. It cannot be concluded, however, that all light naphthenic oils are inherently better than other oil types. The 4 oils used here differed in other factors, e.g. viscosity and distillation range, which may be more important than oil type in causing flecking and disease control. The results from this test only indicate that light naphthenic oils may be superior to other oil types and that this possibility should be investigated further.

More studies are necessary before the characteristics of the best possible oil for spraying bananas can be determined. The present results suggest that future experiments should be conducted using known low-volume deposit rates and emphasizing the testing of saturated fractions from light naphthenic oils.—Federal Experiment Station, Agricultural Research Service, United States Department of Agriculture, Mayaguez, Puerto Rico.

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MECHANISMS OF INHIBITION OF FUNGI BY FILIPIN

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SUMMARY

Exposure of *Saccharomyces cereviseae* to filipin resulted in entrance of the antibiotic into both the cell wall and cytoplasmic fractions. The presence of exogenous sterol reduced but did not prevent antibiotic accumulation in the cell. Filipin inhibited both aerobic and anaerobic respiration of glucose, acetate, and endogenous carbon by yeast cells. In contrast, respiratory activities of cell-free homogenates of the yeast were refractory to the antibiotic under aerobic or anaerobic conditions, and oxidation of glucose, glyceraldehyde, or any Krebs cycle intermediates was not affected by 100 μg/ml filipin. The antibiotic caused a loss in dry weight, as well as the leakage of nitrogen, phosphorus, and nucleic acid compounds from the yeast. Leakage of amino acids was from the cell pool and not due to breakdown of yeast protein. The presence of exogenous ergosterol or cholesterol prevented any of these toxic effects. In aqueous solution, an unstable complex is formed between the sterols and filipin that can readily be broken by the addition of organic solvents. Nevertheless, filipin entered the yeast even in the presence of exogenous sterol. Formation of the complex could not explain the antagonistic action of some sterols. The ability of sterols to complex with the antibiotic was not related to their inhibitory effects on filipin toxicity to growth. Furthermore, cholesterol could reverse the action of filipin even after yeasts had been exposed to the antibiotic for as long as 24 hours; a lag of about 12 hours after sterol addition was necessary before growth began. Filipin alone prevented growth for the entire period of the experiment, and cholesterol alone had no effects. The addition of filipin to yeast incubated in 0.1*M* acetate - 0.01*M* phosphate caused a reduction in the ergosterol fraction that requires hydrolysis prior to extraction by organic solvents.

Previous papers (7, 8, 9) described the inhibition of fungi by filipin and substantiated preliminary observations that some sterols protect fungi from the toxic effects of filipin. No other reports have been made on the interaction between these compounds. There is, however, ample evidence that sterols are essential for the growth of some microorganisms such as protozoa (2, 3, 4), fungi (5, 15), yeasts grown under anaerobic conditions (1), and a few bacteria (5, 6, 14). A study of the antagonistic effect of sterols on inhibition by filipin might help elucidate

both the mechanism of antibotic action as well as the role of sterols in the metabolism of such microbes.

Materials and methods.—Since this report includes data from many different experiments, only the more generalized techniques used are described here, with other techniques and deviations from general procedure deferred to the particular experiments to which they pertain.

For growth studies, 5 ml of Difco Yeast Nitrogen Base Dextrose (YNBD) medium 6.7 g base per 100 ml plus 1% dextrose, in 16-mm test tubes was inoculated with 0.05 ml of a 24-hour liquid culture of *Saccharomyces cerevisiae* Meyen. The organism had originally been isolated from Fleischmann's baker's yeast grown in similar medium. The inoculated tubes were then incubated on a rotary shaker at 25°C, and growth was measured at desired intervals by optical-density readings or per cent light transmission on a Lumetron Spectrophotometer at 540 mμ. Triplicate tubes were always used in such experiments. In experiments with sterols and filipin, all tubes contained equal amounts of alcohol, at total concentrations of 1% or less. Control tubes were always included, containing only alcohols or only sterols. Filipin was added in absolute methanol, and sterols in 95% ethanol.

When the effects of filipin and cholesterol on either oxygen uptake or carbon dioxide production were compared with their gross effects on growth, the conditions maintained were similar to those used for the growth studies. Each Warburg flask contained 3 ml of the complete YNBD and 1.4 mg, dry weight, of yeast from a 24-hour culture. For respiration studies with whole cells, Fleischmann's baker's yeast was washed 3 times with distilled water and suspended in buffer. Amounts of yeast per flask varied from 2.0 to 2.5 mg, dry weight. One ml of 0.05*M* or 0.1*M* substrate was used per flask. Filipin was added in methanol at concentrations that did not exceed 1% alcohol in the final mixture. Total volume was made up to 3.0 ml with potassium phosphate buffer (pH 6.0) at concentrations from 0.03 to 0.1*M* for different experiments. Two-tenths ml of 20% KOH was placed in the center-well of the flasks used for measuring oxygen uptake. Anaerobic carbon dioxide production was measured under an atmosphere of 95% N_2, 5% CO_2.

Cell-free extracts were prepared by the method of Hochster and Quastel (11). Thirty-five g of fresh baker's yeast plus 200 g of glass beads of 4-mm diameter were shaken for 2½ hours in 50 ml of 0.066*M* Na_2HPO_4-KH_2PO_4 buffer (pH 6.0) on a Kahn shaker at 4°C. In experiments designed to measure anaerobic CO_2 production, 1.25 g nicotinamide was added to the buffer. For aerobic studies, the buffer was saturated with lactose (17 g per 100 ml). Resulting cell breakage was about 50%. Whole cells and debris were removed by two centrifugations at 4°C. The light-brown opalescent extract contained numerous small particles and granules but no whole cells. Warburg flasks contained 0.75 ml of freshly prepared extract, 1.0 ml of 0.05*M* or 0.1*M* substrate, filipin or methanol as required, and sufficient buffer to bring total fluid volume to 3.0 ml. The buffer used in the Warburg flask was the same as that used in preparing the extract.

In leakage studies, fresh Fleischmann's baker's yeast was washed 4 times with distilled water and uniformly suspended in an incubation medium. Aliquots of this suspension were distributed to flasks or tubes, and filipin in methanol or cholesterol in ethanol was added to produce various concentrations. Control flasks containing equivalent amounts of alcohol were always included in the experiments, and the total concentration of alcohols was maintained at or below 1%. These suspensions were incubated on a reciprocal shaker at 25°C for various periods. The cells were then removed by centrifugation and the supernatant liquid was decanted. The cell residue was dried for 48 hours at 103°C in tared tubes, and weighed. Amino acid nitrogen in the supernatant was determined by the quantitative ninhydrin method, and phosphorus by the Fiske-Subbarow method. Trichloroacetic acid (TCA) extractable amino acids were determined after prior treatment of centrifuged cells with 5 ml of 10% TCA at 90°C. This mixture was stored overnight and centrifuged, and the residue washed with 5 ml of cold 5% TCA. The supernatants were then pooled and analyzed by the ninhydrin method. For protein determinations, the residue after TCA extraction was dried 24 hours at 80°C and weighed. Fifty mg of the dried residue was used for determination of nitrogen by the micro-Kjeldahl method. Leakage of compounds containing purine and pyrmidine bases was measured by absorption on the spectrophotometer at 260 mμ.

The amounts of protein, ribonucleic acid and deoxyribonucleic acid that had been synthesized were determined on dried yeasts centrifuged from the experimental cultures. Twenty mg of these yeasts were first extracted for one hour at 24°C with 10 ml of ethanol-ether mixture (3:1 v/v) to remove lipids. Lipid-extracted samples were suspended in 2 ml 10% TCA in test tubes, with glass marbles as caps, and heated 45 minutes at 70°C. This mixture was then separated by centrifugation into supernatant and pellet.

For protein analyses, the pellets were resuspended in 10 ml 0.2*N* NaOH and heated at 100°C (5-10 minutes), as required to dissolve. To 0.2 ml of this treated sample were added 5 ml of protein reagent (0.1*N* sodium hydroxide, 98 ml; sodium carbonate, 4 g; 4% sodium tartrate, 1 ml; 2% copper sulfate, 1 ml), and the mixture was heated 10 minutes at 45°C. After being cooled to room temperature, 0.5 ml of a ⅓ dilution of Folin reagent was added and the sample was immediately mixed. Fifteen minutes later the O.D. was measured at 650 mμ with the red filter of the Lumetron colorimeter. The standard was bovine albumin (range 0-100μg).

Deoxyribonucleic acid assays were made by adding 1 ml of supernatant to 2 ml diphenylamine reagent (recrystallized diphenylamine, 1 gm; glacial acetic acid, 96.2 ml; conc. sulfuric acid, 2.8 ml; 0.175*M* acetaldehyde, 1 ml) and incubating the mixtures 17 hours at 30°C, using marbles as caps. The O.D. was read at 610 mμ and 650 mμ by Beckman DU, and the concentrations of DNA calculated as follows:

$$\mu g \text{ DNA in sample} = \frac{(\text{O.D. at } 610) - (\text{O.D. at } 650)}{0.0027} \times \frac{3 \text{ ml}}{1 \text{ ml}}$$

Ribonucleic acids were measured by diluting 0.2 ml. of supernatant to 3 ml with distilled water. The O.D. was read at 260 mμ and 290 mμ by Beckman DU, and the RNA content calculated as follows:

$$\mu g \text{ RNA in sample} = \frac{(\text{O.D. at } 260) - (\text{O.D. at } 290)}{0.006} \times \frac{3 \text{ ml}}{0.2 \text{ ml}}$$

Experimental results.—*Entrance into cells.*—Since filipin is a surface-active agent, it might inhibit the growth of fungi without ever entering the cell. Data from a number of experiments, however, indicate that the antibiotic actually penetrates the yeast and is present in both the cell wall and protoplasm. Forty g of yeast was suspended in 450 ml distilled water containing 10 μg/ml filipin, (equivalent to 125 μg/g yeast) and placed on a rotary shaker. After 1½ hours, the water was removed by centrifugation, and the cells were then washed 5 times with distilled water, and recentrifuged after each washing. The wet, packed cells were then broken by passage through a French-type pressure cell at 16,000-20,000 lb/sq in. Unbroken whole cells and cell-wall fragments were separated from the protoplasm by centrifugation for 10 min at 1400-1500 G. The residue was washed 2 times under the same conditions. Whole cells were separated from cell-wall fragments by suspending the mixture in distilled water and centrifuging 5 min at 250-300 G. The supernatant was recentrifuged 5 times more under the same conditions, until no whole cells remained mixed with the cell-wall fragments. Finally, the cell-wall fraction was deposited by centrifugation at 1400 G.

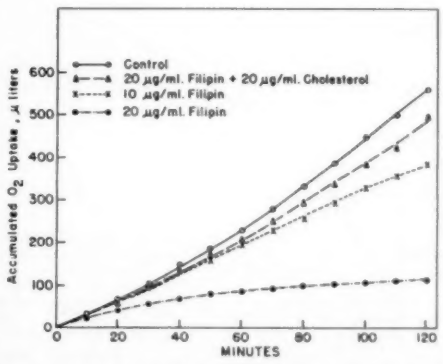

Fig. 1.—Inhibition of oxygen consumption in *S. cerevisiae* cells by filipin and the prevention of such inhibition by the addition of cholesterol to the cell suspension. Endogenous oxygen uptake was 18 μl per hour.

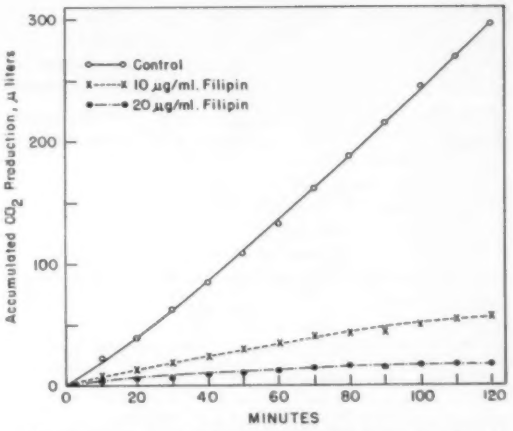

Fig. 2.—Effect of filipin on anaerobic carbon dioxide production by *S. cerevisiae* in YNBD medium. No endogenous carbon dioxide was produced.

Cell-wall fractions were purified by 6 successive washings. Controls were yeasts treated in the identical manner except for omission of filipin from the original suspensions. Both the cell-wall and cytoplasmic fractions were repeatedly extracted with *n*-butanol. Filipin was found in the cell wall and the cytoplasm at respective concentrations of 8 and 1.5 μg/g original dry weight of yeast. The presence of antibiotic in the cytoplasm might still conceivably be due to slight contamination by minute cell-wall particles that had not been deposited during centrifugation; observations in the electron microscope could not rule out this possibility. No absorption peaks corresponding to filipin were found in cells that had not been treated with the antibiotic.

Analyses of whole yeast cells for filipin indicate that the antibiotic enters the cell and that the antibiotic content is proportional to the concentrations in the external solutions. Fifty grams of *S. cerevisiae* were suspended for 1½ hr on a rotary shaker in 450 ml aqueous solutions of various filipin concentrations. Cells were removed by centrifugation and washed 5 times with water; no filipin was found in the last wash water. The yeasts were then extracted 3 times with *n*-butanol, with stirring, and the extracts were combined. At external concentrations of 6.9, 13.8, and 17.3 μg/ml filipin, the antibiotic levels in the yeast were 184, 367, and 471 μg/mg original dry weight of cells. These data cannot be compared to those obtained from cell fractions in the previous experiment because of differences in procedures and loss of cellular material during the fractionation.

Inhibition of respiration.—One obvious mechanism by which filipin could interfere with the growth of an organism is by inhibiting the energy-producing systems. That such a gross effect occurs, is readily seen

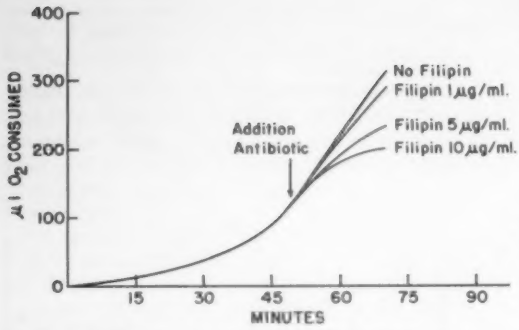

Fig. 3.—Effect of increasing concentrations of filipin on the oxidation of acetate by *S. cerevisiae*. Acetate was 0.05*M* phosphate buffer, pH 7.3.

in a study of oxygen consumption by *S. cerevisiae*. The antibiotic inhibits both aerobic and anaerobic utilization of glucose by freshly grown yeast in either a complete YNBD or a phosphate buffer medium (Fig. 1, 2). Low levels of filipin allow respiration to begin, but the rate of consumption gradually tapers off, then ceases; at the higher levels, oxygen consumption is prevented entirely. Increasing the concentration of filipin progressively decreases the oxygen consumption of yeast cells under aerobic conditions (Fig. 3) and carbon dioxide formation under anaerobic conditions. Respiration measurements in Warburg flasks in the complete growth medium at 0, 10, and 20 μg filipin/ml showed oxygen consumption of 565, 388, and 118 μl, respectively, in two hours. Anaerobic production of carbon dioxide under the same concentrations was 297, 58, and 17 μl. Higher concentrations of filipin, such as 50 μg/ml in a glucose-phosphate buffer medium, completely inhibited carbon dioxide production by *S. cerevisiae*. Oxygen uptake was also completely inhibited under the same conditions, although more gradually. Acetate is readily oxidized by whole yeast cells, and its oxidation was completely inhibited by filipin at 50 μg/ml. The endogenous respiration of yeast grown under anaerobic conditions or of baker's yeast was very sensitive to filipin. At 10 μg/ml filipin, with anaerobically grown cells, the toxic action was apparent within 15 minutes after addition of the antibiotic, and respiration was completely inhibited by higher concentrations of filipin.

Cells that had been exposed to 20 μg/ml filipin in physiological saline for 30 minutes, centrifuged, and washed 3 times with physiological saline solution did not regain their ability to oxidize glucose. Oxygen uptake in control cells and filipin-treated cells was respectively 264 and 4 μl per hour.

Inhibition of cell-free enzyme systems.—Data from many attempts to identify the particular respiratory enzyme systems inhibited by filipin lead to the conclusion that only intact cells are inhibited by the antibiotic. Respiration of whole yeast cells was prevented by the antibiotic, but oxygen consumption was not reduced with cell-free extracts prepared by breaking the yeast either with glass beads or the pressure cell. There was no diminution of carbon dioxide production from glucose by the cell homogenates under anaerobic conditions, even at concentrations of 100 μg/ml filipin (Fig. 4). Similarly, under aerobic conditions, no significant reduction occurred in the oxygen consumption during catabolism of glucose, glyceraldehyde, or Kreb's cycle intermediates (Table 1). This concentration is 50 times as great as is required to inhibit growth of the yeast. Further evidence that the intact cell is necessary for respiratory inhibition was obtained with yeast mitochondria prepared by the method of Linnane and Still (13). No inhibition of oxidation of α-ketoglutarate occurred at 66 μg/ml filipin, which is 4 times that necessary to inhibit the oxygen consumption of whole cells.

Data on the effect of filipin on the respiration of cell-free animal enzyme systems reinforced data obtained with yeast. Rat liver homogenates prepared in glass-distilled water in a Potter-Elvehjem homogenizer oxidized fumarate and pyruvate, but the oxygen consumption was not reduced by the presence of 100 μg/ml filipin. Rat heart homogenates prepared in cold 0.25*M* sucrose in a Ten Broek homogenizer were also refractory to the antibiotic. Inhibition of oxygen consumption with pyruvate and fumarate was only slight, and the decrease was not related to concentration. Rat liver succinic dehydrogenase activity in homogenates remained unaffected by filipin.

Leakage from cells.—Aqueous solutions of filipin cause a decrease in the dry cell weight of *S. cerevisiae*. Five grams (wet weight) of yeasts were suspended in 50 ml of 0.1*M* sodium acetate and 0.01*M* potassium dihydrogen phosphate and incubated 24 hours on a rotary shaker. Filipin was added in amounts needed to produce concentrations of 5-200 μg/ml. Measurable decreases in dry weight occurred

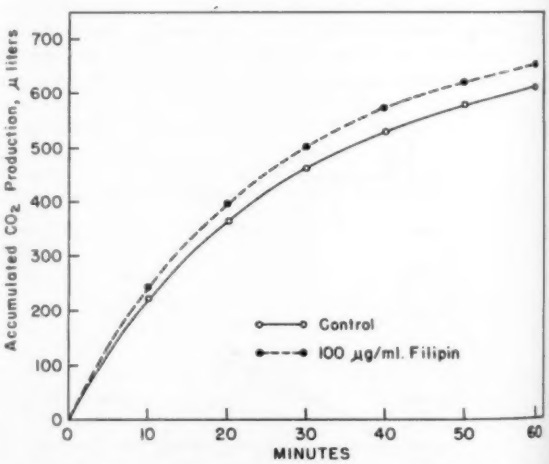

Fig. 4.—Effect of 100 μg/ml filipin on carbon dioxide production by a homogenate of *S. cerevisiae* under anaerobic conditions from 0.04*M* glucose in 0.01*M* phosphate buffer, pH 6.0. No endogenous carbon dioxide was produced.

at 25 μg/ml, with progressively greater losses as the concentration increased until, at 200 μg/ml, the decrease was 35%. A related pentaene, fungichromin, caused similar losses. Further studies made in the absence of phosphate revealed considerable leakage of nitrogen and phosphorus from the cells into the surrounding solution. Leakage from yeasts increased with concentration of antibiotic, and continued over the course of the experiment (Table 2, 3). After 4 hours at 150 μg/ml filipin, 40% of the cell phosphorus and 17% of the TCA-extractible nitrogen appeared in the external fluid.

Qualitative tests revealed the nature of some of the soluble compounds escaping from the interior of the cells to the external fluid. Samples of an incubation medium composed of 0.02*M* glucose and 100 μg/ml filipin, in which baker's yeast had been suspended for 4 hours at a concentration of 0.5 g (wet weight) yeast per 10 ml of solution, was concentrated 20-fold and then used for paper chromatographic analyses.

Compounds exhibiting absorption spectra characteristic of purine and pyrimidine bases move out of the cell. Paper chromatograms of the concentrate of the external solution had 4 absorbing spots that were not filipin. These spots were readily isolated from a papergram developed in an isobutyric acid-water-ammonia (661:329:10) system. The 4 absorbing spots were cut from the papergram and eluted with water, and the absorption spectrum determined. An absorption maximum at 263 mμ, with an inflection at 268 mμ, was found in all the eluates. Other experiments showed that the quantity of UV absorbing material (measured at 260 mμ) moving out of the cell varied with filipin concentration—respectively, 1.5, 26.0, and 69.2 μg/ml at 0, 50, and 100 μg/ml (calculated as guanine hydrochloride).

Ninhydrin-positive materials also escaped from the cell. Chromatography for the ninhydrin-positive material in phenol-water (4:1) containing 50 mg 8-hydroxyquinoline and in 2,6-lutidine-ethanol-water-diethylamine (55:25:20:2) revealed at least 6 spots. One of the amino compounds was identified as glutamic acid. Similar spots were found in yeast

Table 1.—Effect of filipin on oxygen consumption of cell-free yeast systems[a]

Exp.	Substrate	Filipin (μg/ml)	μl O_2/hr[b]
I.	Glucose	0	117
		50	119
II.	Glucose	0	110
		50	117
III.	αketoglutarate	0	213
		50	207
	Sodium acetate	0	116
		50	110
	Sodium citrate	10	261
		50	224
IV.	Sodium citrate	0	86
		100	76
	Sodium succinate	0	387
		100	380
	Fumaric acid	0	134
		100	120
V.	Glyceraldehyde	0	86
		100	83
	Malic acid	0	144
		100	117
	Trisodium cis-aconitate	0	138
		100	130
VI.	Sodium isocitrate	0	180
		100	172
	Oxalosuccinic acid	0	33
		100	48
	Sodium oxaloacetate	0	68
		100	62

[a] Cell homogenates were made by shaking with glass beads. Substrates were 0.05*M* or 0.1*M* adjusted to pH 6.0 to 7.0. Buffer in flask was 0.033*M* phosphate (Na_2HPO_4 and KH_2PO_4) or 0.066*M* phosphate (Na_2HPO_4 and NaH_2PO_4).

[b] No endogenous oxygen consumption occurred in cell-free homogenates.

Table 2.—Effect of filipin concentration on nitrogen and phosphorus loss from Saccharomyces cerevisiae[a, b]

Filipin[c] (μg/ml)	Phosphorus released (μg/ml)	Nitrogen released (μg/ml)
0	1.6	13.8
10	2.8	16.8
20	21.0	40.6
50	31.5	65.1
75	43.7	108.8
100	52.5	157.2
150	66.5	214.7

[a] Per ml suspension: total dry wt = 14.81 mg, total nitrogen = 1250 μg, total phosphorus = 164 μg.

[b] Phosphorus released by 9.8% trichloracetic acid in 4 hr = 113.0 μg/ml.

[c] Analyses were made after 4 hr incubation with filipin.

Table 3.—Course of nitrogen and phosphorus leakage from filipin-treated cells of Saccharomyces cerevisiae.[a, b]

Time (min.)	Nitrogen released (μg/ml)	Phosphorus released (μg/ml)
0	14.7	0.625
15	69.1	6.27
30	72.8	15.75
45	86.8	17.85
60	91.0	19.25
90	114.5	20.65
120	114.8	22.05

[a] Filipin concentration 100 μg/ml.

[b] Total dry wt, 14.43 mg/ml; total nitrogen, 1248 μg/ml; total phosphorus, 163 μg/ml.

extracted 2 hours with 10% trichloroacetic acid, but not in control suspensions in glucose alone. Based on the total ninhydrin-positive material released by treatment with trichloroacetic acid, the amount escaping during a 6-hour incubation in 0.02*M* sodium acetate under the influence of filipin was 17% at 50 μg/ml and 57% at 100 μg/ml. Leakage of ninhydrin-positive material from filipin-treated cells is primarily from the soluble cell pool. Analyses were made for trichloroacetic-acid-extractable material in *S. cerevisiae* treated 6 hours with filipin at concentrations of 0, 50, and 100 μg/ml filipin in 0.02*M* sodium acetate; the respective extracts contained ninhydrin nitrogen equivalent to 80, 66, and 34 μg serine per ml. In contrast, the insoluble fraction from the treated cells analyzed by the micro-Kjeldahl method showed no change in protein content when compared with untreated cells. No sugar phosphate had escaped from the cell to the surrounding medium.

The effect of filipin on sterol synthesis in growing cells could not be determined by gross analysis for free, bound, and total sterol. Despite many trials the data were inconclusive, with the results varying substantially from experiment to experiment. More definite information could possibly be gained from experiments on ergosterol synthesis with cell free yeast enzyme systems.

Synthesis of protein, RNA, and DNA was not reduced in the presence of sublethal concentrations of filipin. The data were obtained from analyses of *S. pastorianus* Hans. on the yeast nitrogen-base dextrose medium at concentrations of 0-6 μg/ml antibiotic.

Sterol-filipin antagonism.—Sterols, such as ergosterol and cholesterol, completely protect fungi from inhibition by filipin. For *S. cerevisiae*, the ratio of Ch/F by weight that allows normal growth is 1:4; even 1:8 shows some reversal. Similar reversal by sterol occurs in the inhibition by filipin of respiration of glucose (Fig. 1) or of the Krebs cycle acids. When fresh cells similar to those used in growth experiments were used to determine oxygen consumption in the YNBD medium, protection afforded by the cholesterol was good at 20 μg sterol and 20 μg filipin. Inhibition was 80% with filipin alone, but only 18% in the presence of equal concentrations of filipin and sterol. The filipin-to-cell ratio used for these respiration experiments was 2 times that normally used in growth studies on a shaker. Thus, under comparable conditions, cholesterol reversal of filipin inhibition of yeast was similar on growth and on oxygen uptake.

The antagonistic effects of cholesterol are also apparent in the prevention of the loss in dry weight of the yeasts and of the leakage of metabolites from the cells. Both filipin and fungichromin caused a weight loss of yeast that was partially prevented by the sterols. Prevention of leakage was still more dramatic. At filipin concentrations of 25-75 μg/ml, nitrogen and phosphorus loss from the cell pool could be completely prevented at Ch/F ratios of 1:1-1:2. Increasing sterol concentration decreased the amount of leakage at any one filipin concentration (Table 4). The prevention of nitrogen leakage was also demonstrated by analyses of cells for trichloroacetic-acid-extractable ninhydrin nitrogen. Results are expressed as μg/ml extract. Untreated yeasts, or those to which 100 μg/ml cholesterol alone had been added, contained 80 μg/ml extract. Cells treated only with 50 and 100 μg/ml filipin had 66 and 34 μg/ml, respectively. Cholesterol and filipin added to the yeast suspension at a ratio of 1:1 (w/w) resulted in nitrogen contents equivalent to that of the control, respectively, 86 and 84 μg/ml at 50:50 and 50:100 ratios.

Table 4.—Nitrogen and phosphorus loss from yeast cells in the presence of filipin and cholesterol.

Filipin (μg/ml)	Cholesterol (μg/ml)	Nitrogen in medium (μg/ml)	Phosphorus in medium (μg/ml)
0	20	10	1
	75	9	1
25	0	24	4
	20	8	1
	40	13	1
	60	11	3
	80	7	1
	100	13	2
50	0	45	12
	20	37	7
	40	35	6
	60	29	5
	80	17	3
	100	8	1
75	0	80	18
	20	79	16
	40	48	11
	60	57	8
	80	27	4
	100	9	0

Leakage of 260-mμ-absorbing material is also reduced by cholesterol, but not to the level of the control. Media from control flasks with the yeasts in 0.02*M* sodium acetate and no amendate or from flasks to which cholesterol had been added, contained after incubation for 6 hours, about 1 μg/ml of purine or pyrimidine bases calculated as guanine hydrochloride. At 50 μg/ml filipin, the external fluid contained 26 μg/ml base, but after addition of sterol at Ch/F of 2:1, only 8 μg/ml were found. Leakage from cells treated with 100 μg/ml filipin was 69 μg/ml, whereas in the presence of the sterol at 2:1 only 20 μg/ml of the bases could be found.

Filipin-sterol complex.—Four types of evidence indicate that no stable chemical bonding occurs in methanol between filipin and cholesterol or ergosterol. First, mixtures of cholesterol and filipin in absolute methanol so that the concentration of filipin was 10 μg/ml and cholesterol-to-filipin ratios were 1:8, 1:4, 1:2, and 1:1 showed no differences in the typical filipin ultraviolet absorption at 305, 322, 338, and 355 mμ. Cholesterol prevented the inhibition of *S. cerevisiae* at all these ratios except 1:8, which gives only partial protection. Second, studies by paper chromatography in 3 solvent systems—water-saturated butanol; 50% acetone and water; and the organic layer of a mixture of benzene, acetic acid, and water

(5:5:1)—revealed no differences in Rf values between the 4:1 sterol-filipin mixture and filipin alone as determined by UV fluorescence and bioautograph. Third, the amount of digitonin-precipitable sterol in a solution remained the same after the addition of filipin as before. A sterol-filipin ratio of 1:10 was used to give a large excess of filipin. The analyses were made by a digitonin precipitation of cholesterol, hydrolysis of the digitonide, and determination of sterol by the Lieberman Burchard method (10). Fourth, a mixture of filipin and ergosterol in methanol, at 1:1 by weight, was stirred for 20 minutes at 50°C, and then placed in a neutral system in the Craig counter-current apparatus for separation of the components. Ergosterol was determined by its absorption at 283 mμ, and filipin at 338 mμ. There was no evidence for the formation of a third component, and both of the original compounds were quantitatively separated, indicating the absence of any stable complex between the two substances.

In aqueous solutions, in contrast, a mixture of filipin and some sterols seems to form a loosely bound complex (12). The effect of cholesterol is illustrated (Fig. 5) because this compound does not absorb UV light. The positions of the UV peaks of filipin are not shifted but the relative intensities change. No consistent changes occur at 322 mμ, but the peaks at 338 and 355 mμ are greatly reduced (Fig. 5). If the reduction in height of the absorption peaks is used as a measure of complex formation with various ratios of antibiotic and sterol, the reaction is not stoichiometric. Disappearance of the 338 and 355 peaks in a mixture of sterol and filipin in distilled water is rapid at first, then proceeds at a decreasing rate until it is essentially complete in about 85 minutes. Complex formation in the growth medium seems to be similar to that in water. The product formed by the sterol-filipin interaction is not very stable, for the addition of an equal volume of methanol to an aqueous solution of the complex results in the reappearance of the typical filipin peaks at about their original intensity.

Further investigations of this reaction indicate that the quenching of the absorption peaks of the antibiotic is not related to the biological activity of the sterols in offsetting the inhibition of fungi. The experimental systems were composed of 5 μg/ml filipin and 5 μg sterol/ml in distilled water, and the reaction time was 2 hours. No drastic changes occurred in UV absorption of filipin with any of the sterols at the 322 mμ peak. At 338 mμ, the absorption by filipin in the presence of the three biologically active compounds was cholesterol, 50%; ergosterol, 87%; and cholestenone, 87% of that absorbed by filipin alone. For the inactive sterols, absorptions were cholestadieneone-3, 92%; androstandiol-3β,17β, 61%; and cholic acid, 94%. In the presence of Δ^4-cholestenol-6β-one-3, which is only about 50% as active as cholesterol, 95% of the original absorption remained. Similar relative values were obtained for the reduction in absorption at 358 mμ.

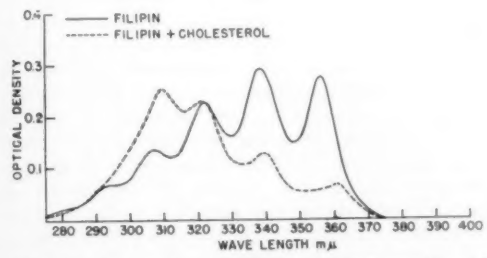

Fig. 5.—Changes in intensity of the UV absorption peaks of filipin in the presence of cholesterol.

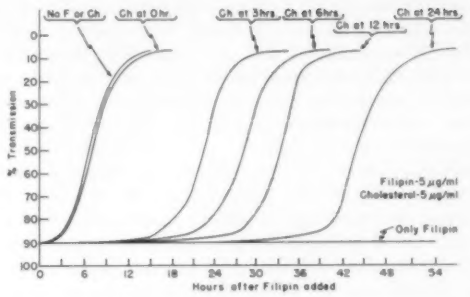

Fig. 6.—Reversal by cholesterol of filipin inhibition of *S. cerevisiae*. Yeast inoculum was added to YNBD medium containing filipin and cholesterol added at various times after the inoculation.

Sterols can vitiate the growth-inhibiting action of filipin not only when cholesterol is present in the medium before the addition of yeast but also if the antibiotic has first been allowed to act on the yeast for a considerable time (Fig. 6). Both the antibiotic and cholesterol were used at 5 μg/ml; cholesterol was added 0, 3, 6, 12, and 24 hours after the filipin. No growth occurred in media that received only filipin, even 54 hours after addition of the inoculum. Optical density in all other tubes eventually reached the same level as those of the controls that did not contain antibiotic or cholesterol. Cholesterol, in the absence of filipin, had no effect. The delay in addition of sterol increased the time required for growth to begin, and was the same for all delayed additions. This delay was 12-13 hours greater than would have been expected if the sterol reversed the toxic action of the filipin instantaneously. Once growth had begun, the subsequent rate of multiplication of the yeast equaled that in the controls, so that the time for maximum growth of the culture after the lag was the same as that for the control. The absence of growth in tubes that received the antibiotic but no sterol is important since it indicates no adaptation to the antibiotic during the experiment, and no inactivation during the long incubation period.

The formation of a filipin-cholesterol complex in aqueous media reduces the amount of filipin entering the yeast cell. Cholesterol and filipin were added to 450 ml distilled water to give the following concentra-

tions: cholesterol, 22 μg in every case, while filipin was 6.9, 13.8, and 17.3 μg/ml. The solutions were allowed to stand for half an hour with intermittent shaking to favor complex formation; they became slightly opalescent and colloidal. Fifty g of baker's yeast was washed and incubated in them for 1½ hours on a rotary shaker at 28°C. Also included were controls of yeast alone, yeast plus filipin, yeast plus cholesterol, and filipin alone. The cell fraction was washed 5 times with distilled water, each time followed by centrifugation, and extracted 3 times with *n*-butanol, and the combined extracts were assayed. In the absence of sterol, the concentrations of antibiotic extracted from the cells at the filipin concentrations of 6.9, 13.8, and 17.3 μg/ml were respectively 183, 367, and 471 μg/mg dry-weight yeast. The respective molar ratios of cholesterol to filipin were 4:1, 2:1, and 1:1. In the presence of sterol, the respective values were 165, 199, and 285 μg/mg dry-weight yeast. Thus, even at the ratio of 4:1, which is 16 times that required to overcome the inhibitory effect of filipin, the antibiotic was bound by the cell despite the formation of the complex.

The antibiotic that entered the cell in the presence of cholesterol was distributed between the cell wall and cytoplasm so that the greatest amount was found in the cell wall. An experiment was described earlier on the occurrence of filipin in the cell wall and cytoplasm; a similar series of flasks was included, containing cholesterol and filipin at a ratio of 4:1 by weight. The cell fractions were separated, extracted, and assayed as previously described. Three typical polyene uv peaks served to identify filipin in the extract. The cell-wall fraction of yeasts from solutions of filipin alone had 3 times the amount found in cells incubated in the presence of sterol—respectively 8.0 and 2.25 μg/mg dry weight of the original yeast. Antibiotic in the cytoplasm was similarly reduced by cholesterol, from 1.5 to 0.5 μg.

The effect of filipin on ergosterol in yeast.—The antagonism of some sterols to the inhibitory action of filipin raises the question whether the antibiotic might affect the ergosterol in yeast. Two forms of ergosterol were studied, "free" which is directly extractable with some organic solvents and "bound" which requires hydrolysis prior to extraction. *S. cerevisiae* was grown anaerobically and then incubated under aeration for 24 hr in 0.1*M* acetate-0.01*M* monopotassium phosphate medium containing various levels of filipin. The cells were harvested by centrifugation and free sterol was extracted by refluxing for 24 hr with 4 changes of ethanol-ether (3:1, v/v), a total of 210 ml of solvent. The extracts were dried, saponified with 10*N* sodium hydroxide, and extracted with ether in a liquid-liquid extractor. The ether solutions were evaporated, the residues were each dissolved in 5 ml hot absolute methanol and were transferred to a 15 ml graduated centrifuge tube. Five ml of a 0.5% digitonin in 50% ethanol solution was added to each centrifuge tube and the tubes were placed at 2°C for 12 hr. The precipitates were centrifuged, were washed with 70% ethanol and were then dissolved in hot alcohol. The yeasts that had been extracted with ethanol-ether were saponified for 2 hr with 10 ml of 10% potassium hydroxide and were then extracted 3 times with a total of 30 ml ether. The digitonides of the "bound" sterol were prepared from the ether solutions as described above for "free" sterol. The concentrations of both types of sterols were determined by the optical density of the UV absorbing peak of ergosterol and the extinction coefficient of ergosterol digitonide.

Table 5.—The effect of filipin on free[a] and bound[b] ergosterol in *S. cerevisiae*.

Yeast fresh wt. (g)	Filipin (μg/ml)	Ergosterol extracted (mg)	
		Free	Bound
7.3	0	4.60	3.81
7.2	25	3.04	0.63
7.5	50	2.16	0.10
7.3	100	4.02	0.00

[a] Extractable with ethanol-ether (3:1, v/v).
[b] Yeasts refluxed with 10% KOH after free sterols were removed, then extracted with ether.

Data in Table 5 show that bound sterol of yeast tends to disappear in the presence of filipin. At 25, 50, and 100 μg/ml filipin, the respective loss was 85, 91, and 100%. The "free" sterol content varied between 47 and 87% of yeast that had not been incubated with filipin but their concentrations could not be correlated to filipin concentration.

Discussion.—Although filipin prevents aerobic and anaerobic glucose catabolism, the initial locus of interference with the metabolic activities of fungi probably is not one of the common respiratory enzymes. The first site of attack is probably the cell wall-cell membrane unit. Cell-free yeast preparations of *S. cerevisiae* carry on aerobic and anaerobic breakdown of glucose and the oxidation of glyceraldehyde and all members of the citric acid cycle, even in the presence of concentrations of filipin that are 50 times that required to inhibit growth. Filipin apparently localizes in the cell wall, whereas only small amounts have been found in the cell cytoplasm; it could thus alter cell permeability to allow the leakage of essential cellular constituents. Morphological changes due to filipin also support this concept (9). Yeasts grown in sublethal concentrations of the antibiotic did not separate into the normal individual cells; the newly formed cells remained attached, to form a mycelium-like thallus. In the absence of nutrients, suspensions of yeast tended to stick together and form clumps of nonproliferating cells. Further, dramatic evidence for cell wall involvement is the formation of swellings on the walls of *Rhizopus nigricans* treated with filipin.

Filipin is a surface-active compound, and its mechanism of action might resemble those of other detergents. With ionic detergents, there is first an adsorptive process that depends on the charges at different sites on the cell surface and on the ionic charge and strength of the inhibitor. Since filipin is a neutral lactone, its attachment to the cell wall does not involve electrostatic forces. Very little is

known about the actual changes in the cell wall brought about by detergents to allow leakage. Some evidence indicates that anionic detergents remove phospholipids. In the case of filipin, however, it seems more likely that any effect on the cell wall or membrane is more likely to involve sterol complexes.

The antagonistic effect of exogenous sterols, such as ergosterol or cholesterol, on inhibition by filipin can be seen in their prevention of leakage from cells as well as in the resultant effects on respiration and growth. If one postulates that the antibiotic disturbs cell-wall integrity by disrupting lipoprotein or lipopolysaccharide constituents and altering the sterol bonding, then exogenous sterol might repair the permeability of the cell wall by replacing the native sterol. A corollary might then be suggested: that sterols play an important part in regulating the permeability of the cell wall membrane.

On the other hand, as suggested by Lampen *et al.* (12), the effect of the sterol in offsetting antibiotic activity might be due only to an *in vitro* reaction between filipin and exogenous sterol. Filipin would thus be prevented from ever reaching the cell. Militating against this assumption is the fact that, even when the sterol-filipin ratio is high and sufficient time is allowed for the complex to form before the yeast is added, filipin enters the cell. An equilibrium must then exist between complexed and free filipin, a situation that is not unexpected in view of a lability of the complex such that even the addition of alcohol breaks the bonding. Also arrayed against the *in vitro* filipin inactivation viewpoint are data indicating no correlation between the ability of compounds to form filipin complexes and their antagonistic action on filipin inhibition of yeast. On the basis of *in vitro* antagonism, it is difficult to explain the effect of cholesterol in promoting the growth of cells that had been inhibited for many hours before the sterol was added. In this situation, we know that filipin enters the cell wall. To allow growth, sterol must also enter and offset the antibiotic effect. Nevertheless, one must recognize that an equilibrium could exist between filipin in the cell and that in the growth medium. As the antibiotic in the external *milieu* is complexed by the sterol, more filipin might be released by the cell, until all is removed and filipin concentration in the cell is decreased to a point at which it is no longer inhibitory. However, suspending filipin-treated yeasts in solutions of sterols did not release the antibiotic.

The possibility must also be considered that the lag period between the time cholesterol was added to reverse the inhibition by filipin and the period at which growth was detected was due to another cause. One could postulate that the antibiotic kills almost all the cells leaving very few survivors. With so few viable cells, a long period of growth would be required before the density of cells in the culture reaches a level that could be detected by light transmission. In this instance one would expect a progressively longer lag period with increasing time of exposure of the yeast cells to filipin before the cholesterol was added. Fewer cells would remain viable at the longer exposures and these cells would take more time to grow to a concentration where growth could be detected. No such increasing lag period was observed.

Some of our data cannot yet be readily rationalized into the main body of information. For example, there is no correlation between the total sterol content of yeasts and the susceptibility of such fungi to inhibition by the antibiotic. Furthermore, growing yeasts in the presence of cholesterol does not subsequently endow them with resistance to the antibiotic.

Another phenomenon requiring more study is the observation that respiration is inhibited in whole yeast cells but not in cell-free preparations. If the present hypothesis of filipin action is correct, this phenomenon might be explained by the reduced concentration in the cell of inorganic ions and other cofactors essential to respiration due to leakage through the cell membrane. A second possible explanation has recently been brought to our attention. In making cell-free enzyme preparations, high concentrations of cells are used. The ratio of filipin to cell material would then be lower than in the studies with whole cells. Thus, inhibitory levels of antibiotic in the cell-free experiments might not have been reached even though concentrations were 50 times greater than that required to inhibit growth of *S. cerevisiae*. Further studies based on filipin-protein ratios should clarify this problem.

It is interesting that filipin inhibits fungi but not bacteria. Fungi synthesize sterols, but only a few bacteria contain such compounds. The ineffectiveness of filipin or fungichromin on bacteria might be due to the fact that sterols do not play as vital a role in their metabolism or in their structure as in the fungi. —Department of Plant Pathology, University of Illinois, Urbana.

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PHYTOPATHOLOGICAL NOTES

A SIMPLIFIED TECHNIQUE FOR HATCHING TESTS OF HETERODERA SCHACHTII

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Hatching tests with cyst-forming nematodes have been conducted in a variety of containers, ranging from petri dishes (1, 8) to watch glasses (4, 6, 7) to hanging drops on microscope cover slips (2, 5). Perhaps the most widely used method (4) employs watch glasses with the cysts lying at the bottom of about 1 ml of hatch solution. The poor aeration under these conditions is not conducive to the best hatching during the early phases. Shepherd (9) has corrected this weakness by employing a device suspending cysts upon a nylon sieve and keeping the cysts wet through a cotton wick to the solution below. This less simple device requires more manipulations resulting in greater expenditure of time/replicate. A desirable procedure would incorporate the best features of these systems and require a minimum time expenditure/replicate. The method described here is a step in this direction.

New System.—A well, approximately 11 mm in diameter, was drilled into a solid piece of plexiglass. A hemispherical screen cyst carrier was formed with a steel punch and dye from a disc of 80-mesh, stainless-steel screen. The hemisphere (8-9 mm diameter), fitting loosely into the well, was prevented from falling into it by a slight shoulder at its rim (Fig. 1). The well depth (depending upon the conical angle of the bit tip) was adjusted so that 0.5 ml of liquid covered the bottom of the screen carrier to a depth of 1 mm. An access hole (about 1 mm in diameter) was drilled to 1 side of the well to form the hypotenuse of a 45° triangle from the apex of the hole left by the bit tip. After hatching, the larvae would emerge from the cysts, pass through the screen, and fall to the bottom of the cell, from which they were removed by inserting a pipette through the access hole. The cysts and cell were washed, the contents removed, and fresh hatch solution added without handling the screen carriers. It was a simple matter to extend this 1 cell to a lattice of cells in a rectangular sheet of plastic.

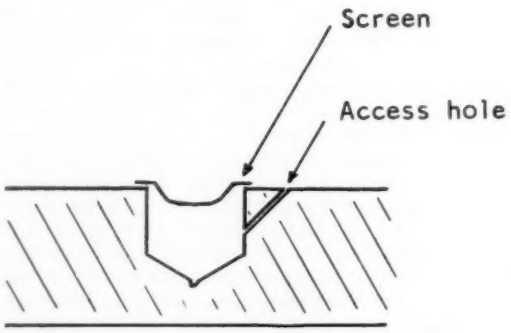

Fig. 1. Schematic representation of the 0.5-ml cell hatch system.

Comparison Systems.—Similar systems, but of differing volumes, 0.25 ml, 2.5 ml, and 12.5 ml, were used for comparative purposes. Cells of 0.25-ml volume were constructed of plexiglass as above, except that filter paper circles were used in place of screens, and there were no access holes.

Embryo dishes were used for the 2.5-ml cells. Stainless-steel screen carriers, larger than those described above, were used to suspend the cysts at the surface of the solution.

Petri dishes (15 x 60 mm) with screen carriers similar to those used with embryo dishes served for 12.5-ml cells.

Lots of about 160 cysts for all systems were measured by volume with a small scoop. The error was less than 5% by weight or number with dry cysts, and less

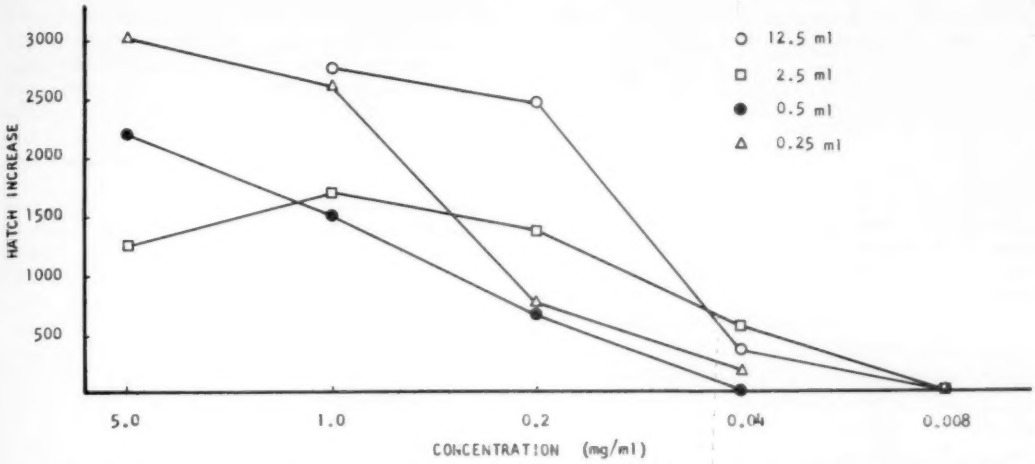

Fig. 2. The curves show the cumulative hatch increase (number of larvae) over water control with increasing concentration of hatch factor at constant volumes of 0.25, 0.5, 2.5, and 12.5 ml of hatch solution at the end of 12 days.

than 10% by number with wet cysts. The cysts were placed directly upon carriers resting on wet filter paper, incubated at 5°C overnight (about 18 hours), then transferred to the appropriate dishes with different concentrations (by a factor of 5) of standard hatch material. The cells were kept in humidity boxes at 25°C, except periodically when larvae were withdrawn for counting and fresh leachate added (about every 4 days).

Fig. 2 indicates the results of the hatch tests at several volumes and concentrations. Similar results were obtained in 5 experiments with 3 different batches of cysts of common ancestry (including fresh wet cysts from growing plants). Each point is the average of 8-10 replicates. By the 3rd collection, larval hatching had dropped to a sufficiently low level not to alter the relative results, and the tests were discontinued. No attempt was made to compare the Shepherd wick (9) to the stainless-steel screen system for aeration effects on hatching. Preliminary tests demonstrated that cysts suspended at the hatch solution surface consistently hatched better than immersed cysts. Since hatching has been shown to be an exponential function of the hatch factor present (3), the semi-log curves should manifest linear portions. The results with the 0.5-ml cell manifested this response more consistently than either the larger or smaller cells. At high concentrations, the crude factor preparation was toxic and hatching was reduced. The 0.5-ml cell provided a satisfactory hatch response. Though the hatch factor concentration may need to be somewhat greater than with larger cells, the smaller volume of solution employed usually requires less total hatch factor than larger cell systems. With the 0.5-ml system, there are no storage problems, fewer manipulations and handling operations, and consequently fewer opportunities for error. About 1,000 cells/day can be handled by an able technician.

The curve for 0.25-ml cells suggests that this volume could be as effective as the 0.5-ml volume. In the use of 0.25-ml and smaller volumes, however, technical difficulties increase rapidly. The cyst volume approaches the hatch solution volume; and the water associated with presoaked cysts, larger in proportion to the hatch solution, becomes an important factor. The savings in hatch material, an important factor with large volumes, is of lesser consequence; manipulations with these very small cells require more time than with 0.5-ml cells.

In the event of cyst selection for increased uniformity in hatching so that fewer cysts/replicate are necessary, the smaller test cell would be useful. Similarly, if hatch factor material were sufficiently precious, the smaller test cell would be advantageous.

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THE USE OF CHENOPODIUM AMARANTICOLOR IN THE STUDY OF NIGERIAN COWPEA YELLOW MOSAIC VIRUS

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Two local lesion hosts, Bengal Bean (*Mucuna aterrima* Holland) and French Bean (*Phaseolus vulgaris* L.) have already been reported for cowpea yellow mosaic virus (1), but neither host is wholly satisfactory for routine quantative assays under temperate greenhouse conditions. A number of additional plants were tested, but only *Chenopodium amaranticolor* Coste & Reyn, reacted to inoculation. Two types of symptom were observed; 1st, local chlorotic spots approximately 1–2 mm in diameter appearing within 4-6 days of inoculation (Fig. 1, left); 2nd, systemic yellow flecks and spots, leaf buckling, and distortion (Fig. 1, right), appearing 5–9 days after inoculation. *C. amaranticolor*, of course, has been widely used as a test plant for many plant viruses (2, 3). This note reports its use as a local lesion host for cowpea yellow mosaic virus.

Inoculations were made in a greenhouse maintained at 21-32°C depending on external weather conditions. Nigerian cowpea yellow mosaic virus was supplied by D. G. Robertson, Department of Agricultural Research, Ibadan, Nigeria, from a greenhouse culture originally established by the author in 1958. The virus was maintained in the greenhouse by serial transfers in cowpea (*Vigna unguiculata* L. (Walp.) var. "New Era"). Cowpea and *Chenopodium* plants used in the investigations were raised in an insect-proof greenhouse maintained at about 21°C. Some inoculations were made using crude sap inoculum; with others, the inoculum was clarified by freezing and then heated to 45°C for 5 minutes followed by low-speed centrifugation. Inoculations were made by dipping a forefinger in inoculum and rubbing it over the upper surface of leaves. "Celite" powder was used as an abrasive and increased the number of lesions. Inocula were distributed among the leaves of a batch of plants so that each was applied equally on different leaf positions. All dilutions were made in distilled water.

Thermal inactivation point.—Samples of infective crude cowpea sap (diluted 1:10 and heated for 10

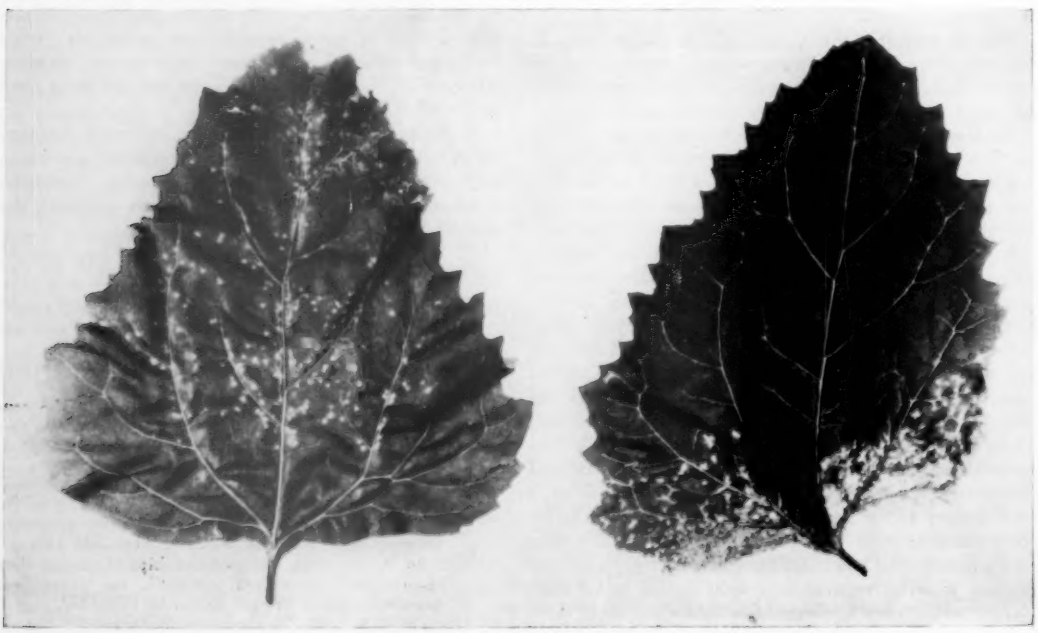

Fig. 1. Left, local lesions on leaf of *Chenopodium amaranticolor* 6 days after inoculation with cowpea yellow mosaic virus. Right, systemic spots, flecks, and leaf distortion on leaf of *Chenopodium amaranticolor* 10 days after inoculation.

TABLE 1. The effect of pH on the infectivity of cowpea yellow mosaic virus assayed using *Chenopodium amaranticolor* (total lesions on 8 leaves)

pH	Infectivity of supernatant	Infectivity of resuspended sediment
2.5	—	—
3.4	8	30
4.6	110	15
7.0	520	48
8.8	590	46
10.0	492	2
Untreated sap	460	—

minutes at 60°, 65°, 68°, and 70°C) produced, respectively, 242, 125, 15, and 0 lesions/leaf when inoculated on *C. amaranticolor;* unheated sap produced 250 lesions/leaf.

Dilution end point.—Infective crude cowpea sap was diluted to various levels and then inoculated on *C. amaranticolor* and French bean (var. "Comtesse de Chambord"). Dilutions of 1:10, 1:100, 1:1000, 1:10,000, and 1:20,000 produced, respectively, 300, 250, 78, 17, and 3 lesions/leaf in the former host; and 145, 91, 21, 0, and 0 lesions/half-leaf in the latter. Therefore, at equivalent dilutions more lesions developed on *C. amaranticolor* than on French bean.

Effect of pH.—Infective sap was frozen, thawed, heated to 45°C, and then clarified by centrifugation. The pH of the supernatant was adjusted to a range of values by adding 0.01 *M* HCl or NaOH. The preparations were centrifuged for 8 minutes at 2,400 *g* and the sediments were taken up in the original volume of distilled water. The resuspended sediments and the supernatant were diluted to 1:100 and then assayed on *C. amaranticolor* (Table 1). At all pH values except 3.4, the infectivity of the supernatant was greater than that of the resuspended sediment.

Precipitation by chemicals.—Infective sap was again frozen, thawed, heated, and clarified before treatment with various percentages of ammonium sulphate, acetone, and ethyl alcohol. The preparations were centrifuged; the precipitates were taken up in distilled water, diluted 1:100, and assayed on *C. amaranticolor* (Table 2). No infectivity was recovered from preparations precipitated with a 20% saturated solution of ammonium sulphate and comparatively little was recovered from preparations precipitated with 20% solutions of acetone and ethyl alcohol. A 66% saturated solution of ammonium sulphate and 66% solutions of acetone and alcohol had no deleterious effects on the infectivity of the virus.

As for so many other viruses, *C. amaranticolor* is a good host plant for cowpea yellow mosaic virus. Sufficient local lesions are produced at inoculation to minimize errors, whereas lesions themselves are large enough to be counted easily. *C. amaranticolor* is easy to raise and plants remain susceptible to cowpea yellow mosaic virus for a long time.

Compared with *P. vulgaris*, the only other readily available local lesion host for cowpea yellow mosaic virus, *C. amaranticolor* not only gives more easily discernible lesions, but also gives more of them at equivalent dilutions of virus. *C. amaranticolor* appears to be the more sensitive host.

Using *Chenopodium*, it was possible to find out something more about the properties of cowpea yellow mosaic virus. For instance, all infectivity was lost in sap which had been adjusted to pH 2.6, although at pH 3.4 there was a little infectivity, mostly in the sediment. Infectivity of the supernatant was maximum at pH 8.8, but even at pH 7.0 and 10.0 it was greater than that of untreated sap. At pH 10.0, there was very little infectivity in the sediment. Cowpea yellow mosaic virus was precipitated from clarified sap by ammonium sulphate, acetone, and ethyl alcohol. Most of the infectivity was recovered from the precipitate using a 50% saturated solution of ammonium sulphate, a 50% solution of acetone, and a 33% solution of ethyl alcohol.

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TABLE 2. The effect of ammonium sulphate, acetone, and ethyl alcohol on the precipitation of cowpea yellow mosaic virus assayed using *Chenopodium amaranticolor*

Ammonium sulphate		Acetone		Ethyl alcohol	
% sat. sol.	Mean no. of lesions on 8 leaves produced by resuspended precipitate	% sol.	Mean no. of lesions on 8 leaves produced by resuspended precipitate	% sol.	Mean no. of lesions on 8 leaves produced by resuspended precipitate
20		20	8	20	15
33	75	33	50	33	125
50	130	50	116	50	135
66	110	66	116	66	170
Untreated	146	Untreated	122	Untreated	140

ANOTHER SCLEROTIAL DISEASE OF WINTER WHEAT IN WASHINGTON

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Groves and Bowerman found *Sclerotinia borealis* Bub. & Vleug. on Gramineae several years ago in Canada (3). The fungus had been reported even earlier from Prince George, British Columbia, by Cormack (1). Recently we found the same fungus on numerous dead and necrotic plants of winter wheat 5 miles west of Chesaw, Okanogan County, Wash., on April 5, 1960. White mycelium and young putty-colored sclerotia were still developing at the edge of melting snow, but most of the sclerotia were dull black. They measured 4-15 × 2-5 mm in diameter and were scattered sparingly over the leaves or nestled in the axils of leaves (Fig. 1-A) and crown parts. They varied in shape from nearly hemisperical to irregularly elongate, especially when 2 or 3 were coalesced. The dead plants were frequently melanistic and some obviously had been dead for several weeks.

The area where the fungus occurs is in the glaciated pine- and brush-covered rolling hills of northern Okanogan County at an elevation of approximately 4,000 ft and subject to relatively deep prolonged snows. At this writing, for instance, on November 29, 1960, the snow at one of our plots near Molson is already 30 in. deep. The yearly precipitation is about 14 in., most of it occurring in late fall, winter, and early spring. Only 5 fields were found infected in a somewhat restricted area from 0.5 mile south of the British Columbia boundary near Molson, Wash., to the field west of Chesaw. The fungus was also found on *Phleum pratense* L. and on *Bromus tectorum* L. The latter was along the roadside 2.5 miles west of Molson. In most fields the *Sclerotinia* was associated with considerable *Typhula idahoensis* Remsb., *T. incarnata* Lasch ex Fr. (*T. itoana* Imai), *Fusarium nivale* (Fr.) Ces., and in some cases *Rhizoctonia* spp. The fields were small although some extended along the sides of hills for considerable distances. The *Sclerotinia* was found in 1 instance at the base of a hill with a south exposure, in other cases on east exposures, and more prevalently on northeast slopes; and 1 field with a southwest slope showed a light infection. The soil was an unclassified dark-colored loam with considerable glacial-broken gneiss and other glacial rock debris in it.

The freshly gathered sclerotia usually failed to germinate on either water agar or PDA, but several cultures were obtained from infected crown parts of wheat and timothy. The cultures grew slowly on PDA at 4°C producing white nonsporulating mycelium. Vari-

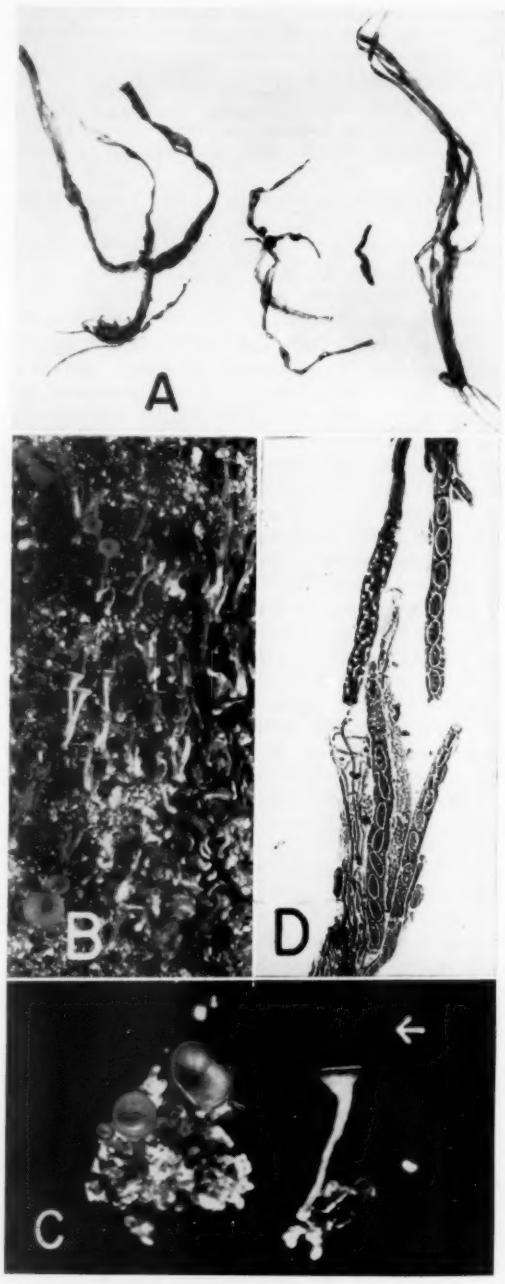

Fig. 1. A) Wheat plants with sclerotia of *Sclerotinia borealis*, near Molson, Wash., April 5, 1960. ×1. **B)** Apothecia of *S. borealis* growing from black sclerotia on wet sand, September 30, 1960. ×1. **C)** Apothecia of *S. borealis* removed from wet sand, September 30, 1960. A pattern of discharged spores (see arrow) formed on the glass mount adjacent to the prostrate apothecium. ×1. **D)** Asci and ascospores of *S. borealis* from field grown ascocarps near Molson, Wash., collected October 26, 1960. ×180.

ous attempts to produce apothecia from newly gathered field grown sclerotia failed under various light and temperature conditions.

Pukhalski (6), however, reported germination of *S. graminearum* Eleneff on cotton wool at 6–8°C after a resting period of 4–5 months. Yakovleff (8) also found that a period of exposure to sunlight is necessary for germination of the fungus. He found that field-ripened sclerotia collected in the autumn germinated 20.5-27.7% while not over 2% of fresh spring-collected sclerotia germinated under similar conditions.

We scattered spring-collected sclerotia over wet sand on August 12, 1960, and kept them under light, including 2 hours per day under a 15-watt germicidal electric lamp held 29 in. above the sclerotia. The sclerotia germinated several weeks later in early September. Mature apothecia were found on September 16, 1960, about 5 weeks after starting incubation at 5-6°C (Fig. 1-B). The apothecia were fawn brown, cyathiform, as large as 9 mm tall, and 4 mm across (Fig. 1-B, C). Apothecia continued to develop for several weeks. When the lid of the incubation dish was removed and the specimens placed under warming light, a cloud of spores was discharged into the air. When several apothecia were removed for photographing, 1 of them, lying flat on a glass slide, produced a spore print (Fig. 1-C). The spores which were mature, or maturing, measured 17-24.2 × 8.2-11.3 μ.

We found mature apothecia in 1 field near Molson, Wash., on October 26, 1960. Apothecia were found in or near wheat stubble and also on *Phleum*. Most of the sclerotia from which they developed lay at or near the surface of the soil in the stubble field. The stipes of the ascophores were as long as 10 mm in cases where the sclerotia were buried. The apothecial cup was usually sessile and rarely extended above the ground surface. The hymenial surfaces measured from 0.5-6 mm diameter. A few were split on the margin as though past maturity. Their color was buff but with some pink color included. The tones varied from a peach tint to some that were more or less tawny.

Fall-seeded plants of winter wheat grown for inoculation purposes in soil in 3-gal stoneware crocks were kept in refrigerated water at 2-3°C. Humidity was held at 95%. Viable field-grown apothecia were placed on the soil surface in 1 crock on October 28, 1960. The fruiting bodies remained fresh for several weeks. Once, when the lid of the cold chamber was removed on November 8, a white cloud of spores arose and drifted over the interior of the chamber. No well-defined lesions had developed on the wheat seedlings by December 3, although some leaf rotting occurred. Black spore masses of *Heterosporium avenae* Oud. very soon covered the lesions. Since, at present, 2°C is the lowest temperature possible with our equipment, we have not been able to pursue the inoculation studies further.

Two species of *Sclerotinia* have been recognized on over-wintering cereals. One, *S. borealis* Bub. & Vleug., was described in 1917 (cf. Saccardo Syll. 24, sect. 2, p. 1179, 1928). Ekstrand (2) used this name. A culture sent to John Hardison by E. A. Jamalainen, Finland, resembles cultures of our fungus. E. J. Hawn sent us a pure culture transfer of *S. borealis* which also originated from Jamalainen's isolations. Lebeau (4) and Lebeau and Logsdon (5) who found *Sclerotinia* on forage crops in Alaska and Yukon used the name *S. borealis* as did Groves and Bowerman (3). On the other hand, the Russian workers use *S. graminearum* Eleneff. Solkina (7) believed that the last mentioned was probably distinct from *S. borealis*. Apparently the first Latin description of *S. graminearum* was furnished by Solkina in 1939 (7) which she said was described by Eleneff in 1919. The Russian writers state that *S. graminearum* has yellowish to yellow-brown apothecia. *S. borealis* is considered to have brown to dilute brown apothecia. Our specimens which germinated under lights in the laboratory have fawn brown tones in the apothecia and buff to slightly pink ones in field-grown specimens. Therefore, depending on their environment, our specimens range in color from those like *S. borealis* to ones typical of *S. graminearum*.

The spore size given for ascospores of *S. borealis* is 19-28 × 7-11 μ, that for *S. graminearum*, 16-23 × 7-10 μ (7). The spores of our material produced under artificial lighting conditions are only slightly longer than those of *S. graminearum* but some spores are as wide or wider than the largest ones reported for *S. borealis*. However, ascospores produced from field-grown apothecia occurring naturally near Molson measured 16-23 × 7.0-9.7 μ (Fig. 1-D). Groves and Bowerman state that both Swedish and Canadian specimens had spores slightly smaller than the size given in the original description. They also observe that apothecia produced in culture tend to be more robust than in nature. We would be inclined to accept the measurements 16-23 × 7-10 μ as fairly typical of those of ascospores in the field. Since, therefore, both the spores and the apothecia vary somewhat according to their environment and since these variations cover the descriptive range of both *S. borealis* and *S. graminearum*, we do not recognize any difference in them. Our field material is virtually identical to the description of *S. graminearum*. Our material grown from field collected sclerotia but germinated under lights is nearly identical to the description of *S. borealis* which Groves and Bowerman determined was originally made from studying cultured apothecia. Based on evidence, therefore, we accept *S. borealis* and consider *S. graminearum* a synonym.

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SOME EFFECTS OF ALPHA IRRADIATION ON GIBBERELLA FUJIKUROI

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Radioactivity is a well-known cause of mutation in fungi, and the effects of radioactivity on plant pathogens are of great interest because of the importance of these organisms to the well-being of mankind (1). Of particular interest is the effect that naturally occurring radioactive substances may have on fungi causing plant diseases (2, 4, 5, 6).

Gibberella fujikuroi (Saw.) Wr. (from the U.S.D.A. Northern Utilization Laboratory, Peoria, Ill.) was selected for this work because it occurs in many plant tissues and in many soils, as do radioactive heavy metals. Mutants of the fungus were obtained by pouring microconidia suspended in sterile distilled water on potato-dextrose agar and exposing them to 10 mc of Polonium-210 in a petri dish. Approximately 200 microconidia were used for each exposure to the radiation.

The number of colonies produced by microconidia surviving a given irradiation was compared to the number from nonirradiated microconidia. An exposure to Polonium-210 which killed 90-95% of the microconidia was most effective for maximum production of mutants. The percentage of mutation was calculated from the number of colonies differing in some cultural characteristics from the colonies arising from nonirradiated microconidia. With a kill of 90-95% about 25% of the surviving colonies were different from the nonirradiated colonies in some characteristics.

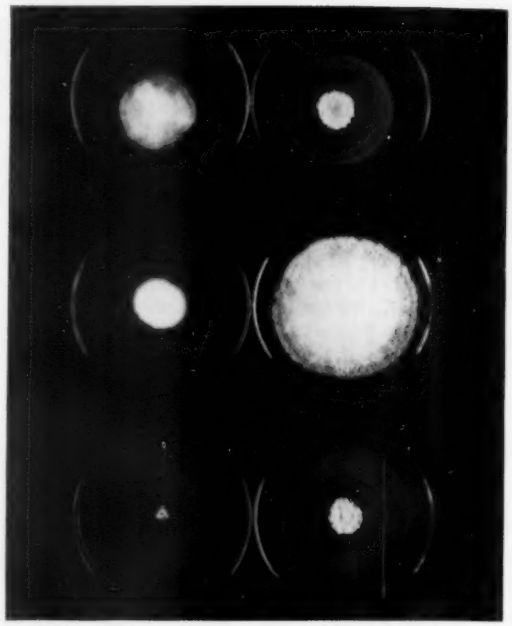

Fig. 1. Mutants of *Gibberella fujikuroi* produced by irradiation with alpha particles from Polonium-210. The parent is in the lower right hand corner.

In general, the mutants differed from parental types in: growth rate, color, colony type, production of gibberellic acid, and pathogenicity to corn. The range in colony type is illustrated in Fig. 1. The production of gibberellic acid was not increased by irradiation.

Twenty-eight of the mutants for growth rate or colony type were compared with the nonirradiated isolate for pathogenicity to seedlings of corn inbred W10 (from the Department of Agronomy and Plant Genetics, University of Minnesota). The corn was sown in 4-in. pots. At the time of planting, 30 ml of a suspension of *G. fujikuroi* mycelium and spores, which had been grown in shake culture for 5 days, was poured into the pots over the seeds and covered with a layer of soil. The pots were placed in a greenhouse at 22.5°C. These experiments were made during winter, 1959. The experiment was performed in triplicate with 5 replications of each isolate in each of the 3 tests, 5 plants in a pot. Pathogenicity was determined by differences in plant height and root rot at the end of 6 weeks.

Table 1 contains average plant height for each isolate. Differences in plant height were found to be significant at the 1% level. The Student-Newman-Kuels Multiple Range Test (3) was performed to isolate nonsignificant groups of means; these are indicated in Table 1 with brackets. None of the isolates caused plants to be significantly taller than

TABLE 1. Average plant heights and root rot ratings for seedling corn inbred W10 inoculated with 28 mutants and the parent strain of *Gibberella fujikuroi* together with the noninoculated check—Brackets enclose nonsignificant groups of means

Isolates	Plant height (cm)	Isolates	Root rot rating[a]
1	40.6	check	4.2
2,3	39.6	7	3.8
4,5	38.6	5,19	3.4
6	38.1	2,6,parent	3.2
check, parent	37.6	1,12,22	3.0
7,8	37.1	4,8,10,11	2.8
9,10,11	35.6	3,9,15,16	2.6
12,13	35.1	13,20,25	2.4
14,15	34.3	14	2.2
16	33.3	23,26,27	2.0
17,18	32.3	17,24,28	1.8
19	31.5	21,18	1.6
20,21	30.5		
22,23	29.9		
24,25	29.2		
26	27.4		
27,28	23.9		

[a] Root rating: 1 = severe rot; 5 = apparently healthy.

noninoculated plants, but 10 isolates caused plants to be significantly shorter than the parent isolate.

Root rot ratings were made on the scale of 1-5: *1*, severe root rot; *5*, roots without root rot. The root rot data are presented in Table 1 and nonsignificant means are grouped with brackets. There were 7 groups of nonsignificant means. None of the isolates was less pathogenic than the parent isolate, but 8 were more pathogenic than the parent.

As can be seen from the Multiple Range Test for both plant height and root rot, groups of means overlap considerably. This would seem to indicate that the pathogenicity of *G. fujikuroi* to corn seedlings may be influenced by a number of different factors and any might be altered by alpha particles.

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QUANTITATIVE PAPER CHROMATOGRAPHIC TECHNIQUES FOR THE ASSAY OF PRODUCTS OF POLYGALACTURONASE ACTIVITY OF FUNGUS CULTURES

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During an investigation of induction of pectic-enzyme forming systems, the enzyme polygalacturonase (PG) which was produced on galactaric acid by the banana-wilt fungus, *Fusarium oxysporum* f. *cubense* (E.F.Sm.) Snyd. & Hans., was found to hydrolyze polygalacturonic acid to monogalacturonic acid (GA) in a system buffered at pH 3.7. Determination of the amount of GA liberated provided an assay of PG activity. Since it was necessary to assay hundreds of single-spore cultures during the investigation, rapid microculture techniques were devised. The present communication reports the use of quantitative paper chromatography to assay for PG in microcultures of *F. oxysporum* f. *cubense*.

Errors inherent in viscometric measurements of PG activity, and in the assay of PG activity on pectinic-acid substrates have been mentioned elsewhere (3, 5). A preferred method of assaying PG activity is to determine the increase in iodine-reducing values resulting from glycosidic hydrolysis of pectic substances (2). Titration of samples containing 1.5 mg. of Mann-assayed GA, however, was found to result in estimates of GA up to 50% over the known amount. Since less than 1.5 mg GA was commonly liberated by PG activity in microcultures, chromatographic assay provided a more reliable estimate of GA than the hypoiodite method. The amount of GA liberated in portions of culture medium-substrate mixtures was estimated by the same methods used for known standards.

In the preparation of cultures, 11 mm × 10 mm (O.D.) microcups were placed in 50-mm petri plates, 15/plate, charged with 0.25-ml liquid synthetic medium, and autoclaved. The cups were then inoculated with single microconidia and the cultures maintained free of contamination within the petri plates during growth. After 3 or 4 days growth, 0.10-ml of 0.5% polygalacturonic acid in HCl-phthalate buffer was added to each culture, and the cultures were incubated with this buffered substrate for 12 hours at 33°C. (If mycelial mats were required, the microcup cultures were dropped into beakers of sterile water, the mats floated out of the microcups, and then transferred by sterile needle to another solution.)

In the preparation of standard chromatograms, 10-μl portions of 0.1*M* GA in culture medium-buffered substrate solution were applied to give spots of uniform size containing 0.25-2.5 μ moles of GA. A 2nd series of spots containing corresponding levels of

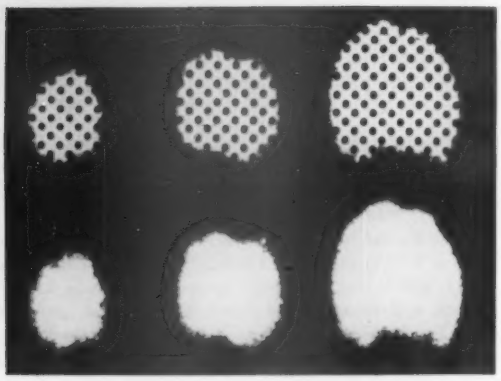

Fig. 1. Spots resulting from chromatographing 0.2, 0.4, and 0.8 mg of galacturonic acid and copying by Apêco autostat, with and without the transparent dotted paper used to estimate spot area.

GA were applied as single 10-μl applications. All spots were applied with centers 2.5 cm from the edge of 28.0 × 21.5 cm sheets of Whatman 3 MM paper. Chromatograms, stapled to form a cylinder, were developed by ascending irrigation.

The solvent and colorization system described by Palmer (4) for other organic acids was modified and used in developing chromatograms. The modified solvent-colorization system contained 50 mg brom phenol blue and 60 mg sodium formate dissolved in a solution mixture of 85 ml of 77% ethanol and 15 ml of 88% formic acid. Following irrigation for 4 hours at 24°C, the paper cylinders were dried and the yellow acid spots and blue background developed by autoclaving for 15 minutes. To prevent water marking during autoclaving, each cylinder was placed upright on a Whatman 3 MM filter-paper disc and another was placed on top of the cylinder. The Rf of GA was 0.52-0.54; polygalacturonic and galactaric acids remained at the origin.

The direct proportionality of spot-area to log content of spot (1) was observed in the range 50-450 μg GA, with known GA dissolved in culture medium-buffered substrate solution, and to 800 μg with GA applied in water. The standard error log content of spot was 20 μg GA, in a sample series of uniformly chromatographed spots containing 360 μg GA. Spot area was estimated by placing uniformly dotted transparent paper (Artype no. 4086) over the yellow GA spots and counting the dots. When several spots were evaluated, it was found convenient to place the transparent dotted paper on a chromatogram and to copy by Apeco auto-stat (Fig. 1).

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RECOVERY OF VIRUS X FROM LEAVES OF GRAFT-INOCULATED IMMUNE POTATO PLANTS

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Contribution No. 50, Canada Department of Agriculture Research Station, Fredericton, New Brunswick. Technical assistance was received from Mr. R. E. Finnie.

The U.S.D.A. Potato Seedling 41956 and some of its progeny—including the varieties Saco and Tawa—are considered immune to potato virus X. Various efforts have been made to test this immunity and the resistance mechanism involved. The virus can remain active for a time in or on leaves inoculated by rubbing (8, 9). It is doubtful, however, if multiplication occurs, and the virus has not been shown to move from the site of such inoculations. But, when a potato scion infected with virus X is grafted to an immune plant, the virus can enter and pass through the immune stem; it can be recovered either from a susceptible basal stalk when the immune stem is the middle section of a double graft (6), or from the roots of the immune plant itself in the case of a single graft (4, 5). Small necrotic lesions are sometimes seen on the leaves of immune plants that have been grafted in this way; these may be due to a hypersensitive-type reaction (7). There has been no direct evidence, however, that systemic invasion of the foliage occurs. I report, here, recovery of virus X from leaves of graft-inoculated immune plants. But, I must reserve judgment as to whether the virus actually multiplies within these plants. The paucity of virus recoveries indicated a low virus concentration and as yet no virus has been recovered from 2nd generation plants propagated by tubers.

For some years I have used the procedure of grafting complex-infected potato material to virus-X-immune potatoes to screen out virus X. The "isolated" viruses—including potato viruses A, F, M, S, and Y, and several "unknowns"—were readily recovered from newly developed shoots of the X-immune stock plants. Occasionally, however, the differential hosts used in these recoveries became infected with virus X. This was first ascribed to contamination, but following an unusually frequent series of such infections (1), de-

liberate efforts were made to recover virus X from graft-inoculated immune plants. Plants of *Datura tatula* L. and *Nicotiana debneyi* Domin., hosts frequently subject to the "contaminations," were used as recovery test plants. These were dusted with 600 mesh Carborundum and rubbed by means of uniform glass spatulas with sap inoculum from immune plants that had been grafted with virus-X-infected scions. The inoculum was prepared by crushing enough leaf material from a single 41956 plant—without petioles—to provide 1 or 2 ml of sap and diluting this with an equal amount of tap water. Test plants that provided 3-6 in.^{2} of inoculating surface on 3 leaves were used; 10 plants were inoculated with each portion of inoculum. For comparison with results obtained by Benson and Hooker (5), I made attempts to recover virus X from roots of some of the same immune plants used for leaf recovery tests. Exposed roots were obtained by placing the pot containing the immune plant on top of another pot which was up-turned over a 250-ml flask of Hoagland's solution. Roots grew through the bottom openings in the pots (enlarged for the purpose) and developed in the nutrient solution. Roots were removed, blotted dry, and crushed to provide sap which was diluted with tap water as before. On each occasion, about 25% of the test plants were used as controls and were rubbed with sap inoculum from uninoculated immune plants. Care was taken to avoid contaminations from the scions or from outside sources—all mortars, pestles, and spatulas were steamed; razor blades, used in excisions, were flamed.

Where test plants were rubbed with leaf inoculum from grafted immune plants, 39 of 280 became infected with virus X. These infections represented portions of inoculum taken from 18 of 28 41956 plants tested, with no more than 4 infections to a portion. Where root inoculum was used, 48 of 120 test plants were infected, representing 10 of 12 41956 plants tested. Two of 130 control plants became infected, both in an early test and probably as a result of some form of mechanical contact in maintenance operations.

To obtain further evidence that the "recoveries" were not, in fact, contaminations, attempts had been made during the above work to introduce into immune plants, different strains of virus X—the "common virus X" from scions of Katahdin potatoes and "virus B" from scions of Irish Cobbler. These are, respectively, the strains that I refer to elsewhere (2) as virus X^x and virus X^{xb}. When virus X was recovered from an immune plant, tests were made to ascertain whether the recovered virus was the strain that had been introduced into that particular plant. If the "recoveries" were really contaminations, discrepancies would be expected. The virus was first transferred from the recovery test plants to tomato plants and scions from the latter were grafted to plants of the potato varieties Katahdin and Epicure. Katahdin reacts with top necrosis to the virus X^{xb} strain, but tolerates the virus X^x. Epicure reacts with top necrosis to both strains.

Where leaves were taken from immune plants that had been grafted with scions containing virus X^x, all 13 tested recoveries were of this strain. These recoveries represented inoculum from leaves of 6 different immune plants and from roots of 3 of these. Where the scions contained virus X^{xb}, all 14 tested recoveries were of this strain; these represented leaf inoculum and root inoculum from 3 different immune plants.

The recoveries of virus X from leaves and roots of immune potatoes have been effected in the greenhouse at Fredericton at different times of the year and, as yet, have not been correlated with any peculiar environment. The less frequent infection of test plants suggests a lower concentration of virus in the leaf inoculum than in the root inoculum. Neither inoculum, however, contained sufficient virus to produce virus-X-type local lesions on the common indicator plant *Gomphrena globosa* L. Sap from neither source gave a positive serological precipitin test with virus X antiserum. Heating the inoculum in hopes of inactivating any heat-labile inhibitor (3) produced no noticeable increase in test plant infections.

I have occasionally observed necrotic lesions similar to those previously reported (7), on leaves of grafted immune plants, and have recovered virus X from such leaves. On one occasion, lesions were observed on foliage of a Saco plant that had been grafted with a scion from Irish Cobbler and tubers of the Saco developed internal necrotic lesions. Similar tuber lesions were reported by Benson and Hooker (5). Such results are in keeping with the suggestion that the immunity to virus X is due to a type of hypersensitive response resulting in extreme localization. The term "hypersensitive response" would imply at least a local infection, though not necessarily multiplication of the pathogen. But even should there be some slight multiplication, resistance here seems adequate to be called "immunity"—"the state or power of resisting the development of a given disease."

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AN EFFECTIVE TECHNIQUE FOR SCREENING SORGHUM FOR RESISTANCE TO CHARCOAL ROT

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Journal Series 159, Agricultural Experiment Station, New Mexico State University, University Park.

In recent years, hybrid sorghums have largely replaced the old standard varieties in commercial production. Because all commercially available hybrids are highly susceptible to charcoal rot caused by *Macrophomina phaseoli*, which increases lodging by weakening the stalks, there is considerable interest in developing resistant hybrids. The search for sources of sorghum resistant to charcoal rot is complicated by the fact that there is no entirely satisfactory technique for inducing the disease in plant material to be screened. The disease does not occur regularly in any 1 location, so that planting the material in infested fields is not a certain test for resistance. Inoculating sorghum stalks with fungus-infested toothpicks, as described by Young (1), is more certain, but does not always induce disease when conditions favor plant development but not the development of charcoal rot inside the stalks. Furthermore, the mechanical introduction of the fungus into the stalks prevents the functioning of any possible natural morphological resistance.

Livingston (2), in Nebraska, found that all 11 sorghum varieties tested in a greenhouse during the summer months of 1943-44 were highly susceptible to charcoal rot. Atlas, which had never shown stalk rot in the field in Nebraska, was 1 of those susceptible varieties. Sclerotia formed readily in the Alliance variety in the 1943 test but slowly in the 1944 test. He attributed this and the similar difficulty of obtaining sclerotia in the greenhouse to difficulty in bringing about normal plant ripening and drying in the greenhouse. Influenced largely by Livingston's findings, a technique for screening sorghum for charcoal rot resistance was tried at this station and it appears more effective than either the natural infection or the inoculating technique. It involves growing of sorghum plants properly spaced in disease-infested soil inside of a greenhouse under extreme heat and under moisture stress during the fruiting period.

PROCEDURE.—Sorghum materials for screening were planted in a charcoal-rot-infested soil bed inside the greenhouse in the summers of 1959-60. In 1959, material was planted in late June; in 1960, it was planted in early July. The top 9-in. layer of the soil bed was made of a mixture of sandy loam soil and small amounts of peat and manure.

The soil was taken from a field south of Clovis where charcoal rot had been severe. The lower layer was native soil, of a loamy clay texture. Seed was planted in furrows spaced 1 ft apart, 5 seeds to each hill, with the hills 8 in. apart. Plants were thinned to 2/hill, 3 weeks after emergence. Dirt was gradually worked to and firmed around the plants as they grew older and taller, to approximate the ordinary field operations of knifing and cultivation. Water was applied abundantly in furrows whenever needed, up to flowering time. From then on, the soil was kept dry to produce a water stress during the fruiting period. One of the 2 plants in each hill was inoculated at the base with an infested toothpick, 2 weeks after pollination. The stalks were split and examined for charcoal rot in November 1959 and in late October 1960, even though severe lodging and outward symptoms of charcoal rot were apparent several weeks earlier.

No accurate recordings were made of temperatures inside the greenhouse. Air temperatures on sunny days, however, frequently exceeded 120°F, even with some ventilation. These plants were subjected to varying degrees of heat stress at temperatures averaging 20–30° F higher than those outside.

RESULTS AND CONCLUSIONS.—In 1959, charcoal rot occurred on all the commercial hybrids tested. Of the plants in 8 replications, 22–78% were infected. Over 90% of the plants were infected with charcoal rot in the summer of 1960. Abundant production of sclerotia was noted in nearly all of the infected stalks. Some experimental strains which were disease-free under natural epidemic conditions also appeared resistant under these conditions. These and other promising materials will be tested further to rule out the possibility of disease escapes.

In most instances, charcoal rot developed in both artificially inoculated and noninoculated plants grown in naturally infested soil inside the summer greenhouse. The spread and intensity of the disease were very nearly the same. Where only 1 plant in a hill was affected, the infection almost invariably occurred on the plant inoculated with the infested toothpicks. If such response occurs in repeated comparisons, it might suggest some morphological resistance to charcoal rot in certain sorghum strains. Otherwise, such difference could probably be explained by the uneven distribution of the natural inoculum in the soil.

Two advantages of this screening method are: 1) high degree of control of heat and moisture stress, and 2) effectiveness in producing charcoal rot regularly. When greenhouse space is limited, this method might be used to screen only material found free of charcoal rot under field conditions of natural infection and

artificial inoculation. Under irrigation, control of moisture stress also may be obtained in fields. Even though heavy, late-season precipitation frequently eliminates water stress during the fruiting period in the open, field screening in disease-infested soil under induced water stress is worth trying because considerably more material can be screened than in the greenhouse.

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THE RELATION OF SEED-TRANSMITTED TOBACCO RINGSPOT VIRUS TO SOYBEAN YIELD

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Cooperative investigations of the Crops Research Division, ARS, U.S.D.A.; and the Purdue University Agricultural Experiment Station. Journal Paper No. 1694, Purdue University Agricultural Experiment Station.

The tobacco ringspot virus (TRSV), reportedly has been transmitted in a high percentage of the seeds on soybean plants grown from infected seeds (2). The small number of seeds on plants that develop from infected seeds suggested that seed transmission might perpetuate and disseminate the virus, but was probably of little importance in development of the bud blight disease. This assumption was based primarily on observation rather than on experimental data. Certification officials and growers of certified seed desired precise information. The effect on yield of planting various proportions of infected seed is reported here.

Seed from TRSV-infected Harosoy plants grown from infected seed was harvested in 1958 and 1959 and planted the following year. The seed from infected plants and noninfected Harosoy seed with approximately the same germination were mixed in proportions from 0-100% at 10% increments. Two hundred seeds were planted in 18-ft rows 38 in. apart, or at the rate of 52 lb/A. Treatments were replicated 4 times in a randomized block. The percentage of infected plants and the seed yield were obtained from the center 16 ft of the row.

The seed from infected plants produced 93 and 88% infected plants in 1959 and 1960, respectively (Table 1). No significant differences in yield occurred until 50% of this seed was in the mixture, resulting in over 30% infected plants. Apparently, up to this point, the noninfected plants, because of less competition, were able to compensate for the reduced seed production of the infected plants. This phenomenon in soybeans has been reported previously in relation to both planting rate (1, 4) and disease (3).

Table 1. Effect of planting various proportions of tobacco ringspot virus-infected soybean seed on yield

% infected seed planted	% infected plants 1959	% infected plants 1960	Bu/A yield 1959	Bu/A yield 1960
0	0	3	39.3	50.0
10	6	9	37.2	47.5
20	12	19	37.1	43.5
30	21	28	37.9	46.0
40	27	34	36.7	43.2
50	38	32	34.4	39.2
60	51	48	32.4	40.8
70	59	56	29.4	37.3
80	69	60	27.2	33.5
90	83	72	24.7	31.0
100	93	88	18.2	23.9
LSD 5% Level			3.1	6.8
LSD 1% Level			4.1	10.7

To determine the percentage of infected plants necessary to produce sufficient infected seed to reduce the yield in the succeeding year, seed harvested from each treatment in the 1959 test was planted in 1960. A significant yield reduction resulted only when seed from 93% infected plants was used (Table 2). In fact, the amount of infection attributable to seed transmission from seed taken from plots with less than 59% infected plants was inconsequential, considering that 3% natural infection occurred in the plot planted from 100% noninfected plants.

Harvesting seed fields with approximately 60% infected plants is unlikely because infected plants remain green until killed by frost; this makes com-

Table 2. Effect of planting seed from various proportions of tobacco ringspot-infected soybean plants on infection and yield

Infected plants in seed source (%)	Infected plants (%)	Yield (Bu/A)
0	3	47.5
6	4	44.7
12	5	44.4
21	5	45.6
27	5	46.1
38	6	46.2
51	5	44.5
59	12	44.4
69	10	43.6
83	12	43.1
93	26	40.8
LSD 5% Level		5.1
LSD 1% Level		7.8

bining almost impossible. Therefore, there is little justification for a certification tolerance based on number of infected plants if yield in the succeeding crop is the sole consideration.

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BOOK LISTINGS

Principles of plant breeding.—*R. W. Allard.*—1960. John Wiley and Sons, New York. 485 p. Price $9.00.

This volume is primarily a textbook for undergraduate students who have had an introductory course in genetics. It contains 36 chapters under nine sections. An introductory section of 5 chapters is followed by 4 sections (20 chapters) dealing with the genetic bases and methods of breeding self- and cross-pollinated crops. Three chapters are devoted to a discussion on breeding for disease resistance, covering such subjects as variation in pathogenic fungi, variation in disease reaction of host species, and factors involved in breeding disease resistant varieties. The last 3 sections deal with the effect of polyploidy on breeding, use of interspecific hybridization in breeding, and mutation breeding.

Vegetable diseases and their control.—*Charles Chupp and Arden F. Sherf.*—1960. The Ronald Press Co., New York, N. Y. 693 p. Price $12.00.

In words of the author, this book is "written in as nearly a nontechnical manner as the facts permit." It is apparent at once, however, that some knowledge of disease causation is assumed, because the fundamentals of pathogenicity are not discussed. Instead, the authors go directly to accounts of specific diseases, beginning in the first chapter with seed-rot, damping-off, and wire-stem control. Then comes a chapter on the "general diseases," such as bacterial soft-rot, bacterial wilt, crown-gall, scab, and the Sclerotinia diseases. The next 17 chapters are concerned with bacterial, fungus, and virus diseases of specific crops. The last three chapters are devoted to minor-element deficiencies, nematodes, and soil sterilization. A list of references is given for each disease.

THE PRESIDENT'S COLUMN

The operation of practically all our Society business, except that of the Council, is in the hands of committees. Obviously then, the committees are important to us. What can we do to maintain interest within our committees, stimulate their creativity, and generally facilitate their operation?

The appointment of committees always and logically has been the responsibility of the Council. The President, however, does have the authority to appoint temporary committees to function during his term of office. Such appointments are not common and usually result only when a special need arises between regular Council meetings, *i.e.*, Annual Meetings. Thus, practically all committees are appointed by the Council when it is in session. At the Green Lake meeting, 30 committees were appointed (or authorized) involving some 250 persons, although not 250 different people. In addition, 14 representatives were appointed.

In practice, the Council is provided by the Secretary with a list of current and recent past committee members slightly in advance of the meeting. During the Council meeting and as a result of an hour or 2 of deliberation, the appointments are made. There are 3 main problems: first, the pressure of time; the Council must conduct much business in the short time available. Second, insufficient familiarity with the specific goals and accomplishments of the different committees. Third and most important, lack of knowledge of suitable potential committee material. As might be expected, there is a marked tendency to reappoint the same or essentially the same committee personnel. Furthermore, some members who have demonstrated the ability to get things done on a committee not only are reappointed but may serve on several committees at the same time.

The Council is concerned about this and at the Green Lake meeting asked W. W. Hare, then a Council member, to take the lead in studying this situation and to submit recommendations for improvement. In effect, the Council appointed itself a committee of the whole to improve procedures for appointing committees!

Dr. Hare has submitted a report of recommendations based on a consensus of Council opinion. He points out that there are really no indispensable men, that the Society would get a better job from a given member if he were not overloaded with committee assignments, and that our Society is getting fairly large, in fact 2,093 at last count, which should provide an adequate supply of talent. It is recommended that: no member may serve on a specific committee longer than 3 years; that the chairman of a committee shall have at least 1 year of prior experience on the committee (except for new committees); and that no member shall serve on more than 1 committee at a time, except for ex-officio appointments, the Editorial Board, and the Program Committee. It is understood that under unusual circumstances exceptions would be made.

These 3 basic recommendations should go a long way to revamping our committees and spreading the responsibilities. Under these new plans, about 1 member in 10 should have a current committee appointment.

Problem 1, sufficient time, and in part, problem 2, knowledge of the committee, are to be met by an extended procedure for submitting nominations. Some 2 months ahead of the annual meeting, the Secretary will furnish the Council with a list of current and recently past committees. The Council then will return nominations for committee members and chairmen. Similarly, current committee chairmen will be asked to return nominations for their committee. These various nominations will be collated and given to the Council a month ahead of the annual meeting. In order to provide an essential background for making nominations, however, presumably it also will be necessary for the various committee reports to be in the hands of the Council by this early date. Thus, there should be plenty of time for consideration. The final appointments will be made at the annual Council meetings. It should be pointed out that all of these recommended procedures would, of course, be self-imposed by the Council. With the passage of time and experience, it is to be expected that modifications may be made by the Council as it sees fit.

Problem 3, knowledge of potential committee members, is helped by more time but is still a problem. The Council, even under the best of circumstances, is still circumscribed by the subject matter fields and geographical limitations of its 15 members. How can the Council discover new men, especially in the younger group of the Society, who would be alert and hardworking assets on a committee?

One approach, not included in the above recommendations, would be for the Council to appoint only the committee chairmen. The chairman would be asked to select his new committee subject to Council approval. A chairman wisely selected should have a better knowledge of suitable members than the Council. Such a committee might be expected to work more effectively and rapidly. It probably would be more restricted, however, to the chairman's viewpoint. A less radical approach would be to lean heavily on the chairman's suggestions for replacements at the end of the year as indicated above. In the past, Secretaries have requested such suggestions, but generally have received little or no response from the chairmen. We think that the chairmen should take this request more seriously.

As a final thought, we believe that it would be in order for the members at large to make suggestions to the various Council members for possible assignments to specific committees. Need we emphasize that qualifications for nominees are a real interest and knowledge of the area embraced by the committee, some refreshing ideas, a cooperative and hardworking nature, and in short, for the time being at least, a dedication to our Society.

S. E. A. McC.

NEWS

Editor: K. W. KREITLOW, Plant Industry Station, Beltsville, Md.

The North Central Division meetings will be held June 26-27 at South Dakota State College, Brookings. The meetings will consist of laboratory exhibits, greenhouse and field experiments and some formal discussions, with a banquet and address on the evening of June 26. An invitation is extended to all members of the North Central Division, also, members of other Divisions including Canadian phytopathologists who may be traveling in this area when the meetings are in progress.

Phytopathologist John T. Middleton, Chairman of the Department of Plant Pathology, University of California, Riverside, has been elected Chairman of the State Motor Vehicle Pollution Control Board established to implement provisions of Assembly Bill 17 passed at a special session of the 1960 State Legislature. Members of the board were appointed by Governor Edmund G. Brown in June, 1960. In a pioneer attempt to control motor vehicle exhaust emissions, the Board will establish criteria for testing systems for the control of mobile sources of air pollution, evaluate the effectiveness of control systems, and certify systems which are found acceptable.

The University of Arkansas at Fayetteville was the host institution for the annual meeting of the Southern Region Technical Committee on Plant Parasitic Nematodes (S-19). Representatives from 13 Southern States, Puerto Rico, and the Nematology section USDA met February 16 and 17, 1961, to review the progress and accomplishments of research activities and discuss direction for future work.

Diseases and pests of forage grasses is the subject of an International Symposium to be held June 12-14 at the Institut für Phytopathologie, Karl-Marx Universität, Leipzig. Details of the program may be obtained from Prof. Dr. F. Muhle, Institut Director.

Extension plant pathologist M. C. Shurtleff, Iowa State University, Ames, has accepted a position in the University of Illinois, Department of Plant Pathology effective July 1. Dr. Shurtleff will conduct the extension program in diseases of agronomic and vegetable crops and conduct research on cereal diseases.

The Botany Department at the University of Connecticut recently moved into a New Life Sciences Building. New greenhouses were constructed and are now in operation. Graduate training in the fields of mycology and plant pathology has also been instituted.

Plant pathologist David Gottlieb has been appointed to the Center of Advanced Studies for one year, effective September 1, 1961. The center is a special unit of the Graduate College of the University of Illinois for the encouragement of creative achievement and scholarship by providing recognition to scholars of the highest distinction and by providing incentives for the highest level of scholarly achievement.

The "Results of 1960 Fungicide and Nematocide Tests" has again been printed and is available at $1.00 per single copy. Copies can be secured only from Dr. A. B. Groves, Department of Plant Pathology and Physiology, Winchester Fruit Research Laboratory, Rural Route 3, Winchester, Virginia. All orders should be accompanied by remittances made out to The American Phytopathological Society. An added charge will be made for postage and handling where orders must be billed.

Pathologist F. H. Berry recently of Beltsville, Maryland, has joined the pathology section of the Forestry Department at the University of Missouri to work on tree diseases, particularly oak wilt.

Pathologist W. B. Raymer, formerly assistant professor of plant pathology at Oregon State College, accepted a position with the Crops Research Division of the USDA. Dr. Raymer is located at Beltsville, Maryland, and has primary responsibility for potato disease research.

The Pacific Division of the American Phytopathological Society will hold its 43rd annual meeting June 21-23 on the Davis campus of the University of California.

Organic chemist William Moje has joined the Department of Plant Pathology, University of California, Riverside, as associate chemist to investigate the relationship of molecular structure and mode of action of fungicides, including chemotherapeutants, to plant disease control.

Plant pathologist Leonard J. Alexander of the Ohio agricultural Experiment Station will spend the year July, 1961-62, at the Institute voor Plantenziektenkundig Onderzoek, Wageningen, the Netherlands. Dr. Alexander is the recipient of a Rockefeller grant and will work on the serological relationships which exist between pathogenic strains of tobacco mosiac virus and the hosts tomato and tobacco.

Pathologist E. C. Blodgett has accepted a United Nations assignment to assist and advise Israel in establishing and operating field plant quarantine stations. Dr. Blodgett, on a years' leave from the Washington Agricultural Experiment Station, Prosser, will also train technicians.

A recent graduate of Cornell University Richard Ford has accepted a position in the Crops Research Division of the USDA in Corvallis, Oregon. Dr. Ford is associated with the Department of Botany and Plant Pathology at Oregon State College, and will investigate pea diseases.

Pathologist A. H. Eddins recently spent a month as a consultant on Irish potato production at the Facultad de Agronomia, Universidad Central de Venezuela, Maracay, Venezuela. His assignment was made possible by a grant from the Creole Foundation of Venezuela.

After 40 years' service in the Department as a plant pathologist and a breeder of tomatoes, William S. Porte retired from the Vegetables and Ornamentals Research Branch of the USDA, Crops Research Division, February 28, 1961. Mr. Porte joined USDA in 1918 and is best known for his work in breeding wilt-resistant tomatoes. Mr. Porte received a USDA Superior Service Award in 1955.

PHYTOPATHOLOGY Volumes 1-5, which have long been out of print, have recently been reprinted. Volumes 1-5, 1911-1915, paper bound set $80.00, single volumes, paper bound $16.00. All orders and inquiries should be addressed to: Johnson Reprint Corporation, 111 Fifth Ave., New York 3, N. Y.

Phytopathologist Bert M. Zuckerman will attend the Sixth International Nematology Symposium to be held at the State Agricultural College, University of Ghent (Belgium) July 24-28. He will present a paper on the physiology of parasitism of sheath nematodes. Several weeks will be spent in Europe visiting workers in Holland, Belgium, and England.

Deaths Julian H. Miller, 70 died of a heart attack on March 25 at his home after a short illness. Dr. Miller was the former head of the Department of Plant Pathology and Plant Breeding at the University of Georgia, and since 1958 has served as Professor Emeritus. He had recently completed a monograph on Hypoxylon and was working on a monograph of the Xylariaceae with the exception of Hypoxylon at the time of his death.